Identification and characterization of oncogenic pathways involved in rhabdomyosarcoma

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

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Zürich, 2007

Die vorliegende Arbeit wurde von der Mathematisch-naturwissenschaftlichen Fakultät der Universität Zürich im Sommersemester 2007 als Dissertation angenommen. Promotionskommitee: Prof. Dr. J. Jiricny (Vorsitz), Prof. Dr. B. W. Schäfer (Leiter der Dissertation)

To my parents
and
Stonee

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1 Summary

Rhabdomyosarcoma is a malignant tumor deriving from muscle progenitor cells. Four histologically different subtypes of RMS are known, the most common are the embryonal (eRMS) and alveolar (aRMS) forms. About 80% of all aRMS cases are associated with a specific translocation t(2;13) or (1;13) leading to the formation of a fusion gene, PAX3/FKHR or PAX7/FKHR. PAX3(7)/FKHR is a strong transactivator and has previously been shown to activate a number of antiapoptotic pathways and modify growth and differentiation of cancer cells, thus contributing to the oncogenic behaviour of these cells. Furthermore, recent data indicate that PAX3FKHR target genes are also implicated in the - compared to eRMS much larger - invasive and metastatic potential of aRMS. Direct targets and molecular mechanism of these pathways though are largely unknown; therefore the identification of direct targets of PAX3/FKHR was the main goal of this project.

For the identification of direct PAX3/FKHR targets a microarray approach was chosen. This initial screen was complemented by functional analysis of interesting target genes to elucidate the oncogenic mechanisms of PAX3/FKHR. As cellular model for this screening, the PAX3/FKHR-positive aRMS cell line Rh4 was chosen. The transcriptome of this cell line most closely reflects an in vivo PAX3/FKHR-specific gene signature found in a recent study of aRMS biopsies.

In the present work, endogenous PAX3/FKHR was downregulated by RNAi in Rh4 cells, followed by a gene expression profiling using Affymetrix HGU133A arrays. In accordance with previous studies, analysis of cell growth and apoptosis rate upon downregulation of PAX3/FKHR demonstrated a stong influence of PAX3/FKHR on viability of Rh4 cells. The measured gene expression profiles were then compared with the in vivo expression signatures specific for PAX3(7)/FKHR-translocations. By this analysis, 51 putative target genes physiologically relevant for development of rhabdomyosarcoma could be identified.

One identified target gene, namely TFAP2B, was further characterized as PAX3/FKHR target. First, direct DNA binding of the PAX3/FKHR protein to the promoter of one identified direct target gene, TFAP2B, was analyzed by promoter studies, chromatin immunoprecipitation and EMSA. These methods validated TFAP2B as PAX3/FKHR target. Furthermore, functional studies revealed that TFAP2B acts as an essential mediator of PAX3/FKHR oncogenic properties.

The identification of physiologically relevant PAX3/FKHR target genes is crucial for understanding the oncogenic mechanisms involved in the development of rhabdomyosarcoma. Functional studies of PAX3/FKHR target genes could also reveal novel targets for specific cancer treatment.

2 Zusammenfassung

Das Rhabdomyosarkom (RMS) ist ein maligner Tumor, der aus Muskelzellvorläufern entsteht. RMS werden in vier Subtypen unterteilt, dabei sind das embryonale RMS (eRMS) und das alveoläre RMS (aRMS) am häufigsten vertreten. Etwa 80% der alveolären RMS weisen die chromosomale Translokation t(2;13)(q35;q14) auf, das daraus resultierende Fusionsgen wird als PAX3/FKHR bezeichnet. PAX3/FKHR wirkt als starker Transkriptionsfaktor, dessen onkogener Mechanismus allerdings nicht gänzlich aufgeklärt werden konnten, da erst einige wenige Zielgene charakterisiert wurden.
Es gibt einige Hinweise darauf, dass die Zielgene des PAX3-FKHR für die hohe Rezidivrate und Metastasierung dieser Krebsart mitverantwortlich sind. Daher wurden im Rahmen dieses Projektes solche direkten Zielgene des PAX3/FKHR Fusionsproteins charakterisiert. Zur Realisierung des Projektes wurden zwei experimentelle Ansätze verfolgt, zum einen die Charaterisierung der Zielgene von PAX3/FKHR durch Microarray-Analysen und komplementär dazu die Klärung der onkogenen Mechanismen durch funktionelle Untersuchungen der Zielgene.

In der vorangegangenen Expressionsanalyse von eRMS und aRMS Tumorbiopsien wurde ein für translokations-positive Proben typisches Genexpressionsmuster gefunden. Diese Gensignatur konnte auch weitgehend für die Rh4-Zellen, einer Modellzellinie für aRMS, bestätigt werden. Nun wurde mit Hilfe der RNA-Interferenz Technik die Expression des PAX3/FKHR Gens in Rh4-Zellen vermindert und daraufhin auftretende Veränderungen in der Expression der aRMS-spezifischen Gensignatur untersucht. Veränderungen im Expressionsprofil führten zur Identifikation von 51 Kandidatengenen, die Zielgene der PAX3 und PAX3/FKHR Transkriptionsfaktoren darstellen und daher mit wichtigen phänotypischen Charakteristika dieser Tumorzellen assoziiert sind.

Komplementär zu den Expressionsanalysen wurde die Protein-DNA Interaktion zwischen dem PAX3/FKHR Protein und den spezifisch gebundenen Sequenzen der DNA eines Zielgens, TFAP2beta, mittels Promoteranalyse und Chromatin Immunopräzipitation erforscht, um Rückschlüsse auf die Mechanismen der Zielgenaktivierung ziehen zu können. Diese Methode erlaubte zudem eine zusätzliche Verifizierung von Zielgenen und erlaubte die Untersuchung ihrer Rolle und Relevanz bei onkogenen Vorgängen.

Die Auswirkungen der durch RNA-Interferenz verminderten Expression der PAX3 und PAX3/FKHR Gene und somit veränderte Expression derer Zielgene auf physiologische Prozesse in den eRMS und aRMS Zellen wurden ebenfalls untersucht. Von besonderem Interesse waren hier die Veränderungen in der Proliferations- und Apoptoserate der Zellen, da es einige Hinweise darauf gab, dass PAX3/FKHR Wachstumsfaktoren und anti-apoptotische Proteine aktivieren könnte. Die Zellwachstum fördernde Eigenschaft von PAX3 und PAX3/FKHR konnte bestätigt sowie die Beteiligung von TFAP2beta in diesem Prozess gesichert werden.
Protein-DNA Interaktionen spielen eine essentielle Rolle in allen Bereichen der Genregulation, daher ist die Charakterisierung der Zielgene von PAX3/FKHR besonders wichtig für das Verständnis der onkogenen Mechanismen, die das Rhabdomyosarkom verursachen. Die funktionelle Untersuchung

der Zielgene von PAX3/FKHR könnte in der Zukunft neue Ziele für spezifischere Krebstherapien liefern und wäre für die Entwicklung von neuen Krebstherapeutika von Bedeutung.

3 Introduction

The multiplication of cells is one of the most important and sensitive processes in the body and is therefore carefully regulated. There are various mechanisms and controls to balance these processes of cell birth and death to produce a steady state. For some cell types, such as epithelial cells, there is a rapid renewal, some others rarely die or, as in the case of brain cells, little or no replacement of those cells occurs. Very rarely, the numerous controls that regulate cell multiplication break down and an uncontrolled cell growth and division begins. When such a cell gives rise to a cell population that inherit the propensity to grow without responding to regulation, this may lead to tumor formation. The genetic and physiological events that lead to transformation of normal cells are crucial for tumor formation and thus, the understanding of those processes is very important for diagnosis and treatment of cancer.

3.1 Cancer

Cancer development in humans is a slow process: For most solid human tumors, there is a period of years needed for tumor formation. Cancer research of past decades revealed cancer as a genetic disease involving multistep processes, where each step reflects genetic alterations that drive the transformation of normal human cells into highly malignant cells. A great effort has been done in last years to understand genetic processes which lead to tumor formation and progression.

Already in 1971, a mechanism for tumor formation was proposed by Alfred G. Knudson in his two-hit theory. Analyzing clinical and statistical data of patients with retinoblastoma, he noted that as normal individuals have two normal copies of an tumor suppressor gene, two independent 'hits' (mutations) are required in the same cell which then lead to neoplastic development. Knudson's two-hit theory, first stated for retinoblastoma, turned out to be correct for essentially all cancer types, although it has been shown later that in most cases more than two hits are required for tumor formation. However, recently published findings suggest that also monoallelic single-hit events inactinvating a tumor suppressor gene might be sufficient for development of Wilms tumor if the inactivation occurs on the single X chromosome in tumors from males and the active X chromosome in tumor from females (Rivera et al., 2007). The multistep development of cancer is now a more accepted model of tumorigenesis. The multi-hit hypothesis proposed by Vogelstein and Kinzler in 1993 suggests that multiple mutations (three to six) have to occur over longer period of time in a single cell to form cancer. D. Hanahan and R. A. Weinberg postulated six alterations in cell physiology as hallmarks of cancer cells which are most likely shared by all types of human tumors. These six characteristics of cancer cells are: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis as shown in Figure 1 (Hanahan, Weinberg, 2000).

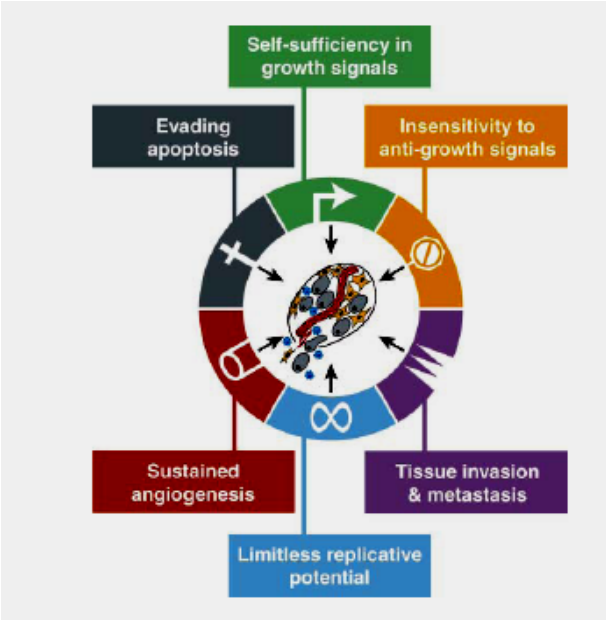
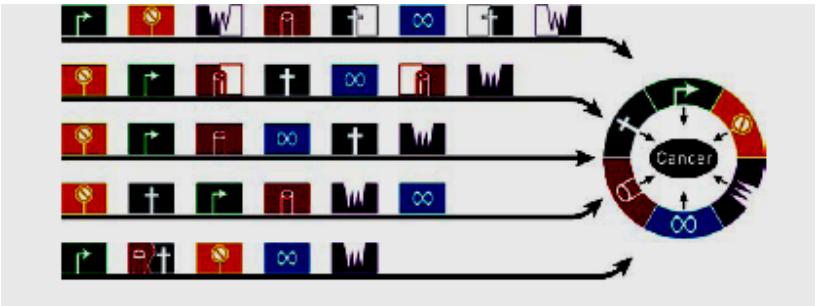


Figure1 Acquired capabilities of cancer (Hanahan, Weinberg, 2000)

The mechanisms how these six hallmark capabilities are acquired by cancer cells differ significantly. There are different pathways involved in tumorigenesis, varying in both, mechanism and chronology. The order in which these tumor characteristics are acquired can vary in different cancer types, as shown in Figure 2.



- Self-sufficiency in growth signals
- Insensitivity to anti-growth signals
- Evading apoptosis
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis

Figure 2 Possible assembly of pathways leading to cancer formation (adapted from Hanahan, Weinberg, 2000)

There is increasing evidence that human tumors are genetically unstable and that most mutations are acquired, directly or indirectly, through changes in the genome of cancer cells. However, the normal mutation rates are insufficient to account for the numerous mutations detected in human cancers, as a complex genomic integrity maintenance system exists in each cell. Therefore, mutations that increase mutation rates and the malfunction of the components of the genomic “caretaker” systems are essential to account for the large number of mutations observed in human tumors. According to this hypothesis, the term of mutator phenotype of cancer cells has been stated for mutations in genes that maintain genetic stability, as genes that control the DNA replication and efficacy of DNA repair. Additionally, mutations in genes that encode for components that maintain normal DNA sequence in cells also contribute to mutator phenotype (Loeb et. al, 2003). The genomic instability which enables acquirement of cancer related characteristics exists at two distinct levels. First, the instability at nucleotide level which involves base substitutions or deletions or insertions of a few nucleotides. Second, the instability at the chromosomal level, resulting in numeral and structural alterations of chromosomal material and therefore gains and losses of whole chromosomes or portions thereof also leads to tumor formation (Lengnauer, Kinzler, Vogelstein 1998). Genes which are responsible for tumorigenesis can be divided into three groups: oncogenes, tumor-suppressor genes and stability genes. Cytogenetic changes and mechanisms leading to alterations in these genes and thus to tumorigenesis as well as most important genes will be discussed below.

3.2 Cytogenetic changes in cancer

Generally, there are three main cytogenetic changes occurring in cancer cell: deletions, amplifications, translocations and inversions (Rabbitts, 1994). Deletions often lead to the loss of tumor-suppressor gene, translocations and inversions can lead to gene activation and to gene fusion, as shown in Figure 3. Gene activation occurs mostly through a translocation or inversion event, joining the gene (proto-oncogene) to immunoglobulin or T-cell receptor gene, thereby activating it. Immunoglobulin and T-cell receptor genes are often involved in chromosomal aberrations because they are naturally rearranged, therefore this gene activation event is observed frequently in leukaemia and lymphomas, as for example the juxtaposition of *c-MYC* and immunoglobulin heavy-chain (H) genes in Burkitt's lymphoma (Rabbits, Boehm, 1991) or juxtaposition of *HOX11* gene and T-cell receptor in T-cell acute leukaemia (T-ALL) (Dube et al., 1991).

A

B

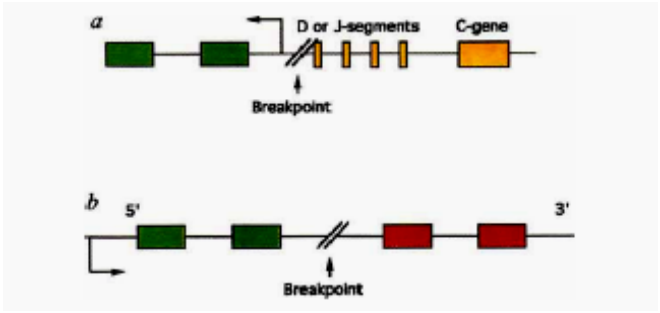


Figure 3 Chromosomal abberations shown schematically for two hypothetical genes. (A) Gene activation by joining of the oncogene (green exons) to diversity (D) or joining region (J) segments, depicted in yellow. (B) Gene fusion of two hypothetical genes (green and red), which became joined as a result of translocation or inversion. Transcription of the resultant fusion gene results in mRNA encoding a fusion protein comprising parts of both green and red protein (adapted from Rabbitts, 1994).

Additionally to the commonly occurring cytogenetic events, a novel mechanism of formation of a fusion gene has been shown recently in T-ALL (Graux et al., 2004). As shown in Figure 4, the fusion of the *NUP214-ABL1* gene is not created by a chromosomal translocation or inversion of interstitial deletions. In this case, both genes *NUP214* and *ABL1* are located at chromosome 9q34 and the fusion is mediated by extrachromosomal amplification which is not detectable by conventional cytogenetic analysis. However, by the use of novel methods like FISH, amplifications or losses of chromosome regions can be detected with high resolution, which is an important tool for diagnosis of cytogenetic aberrations in cancer.

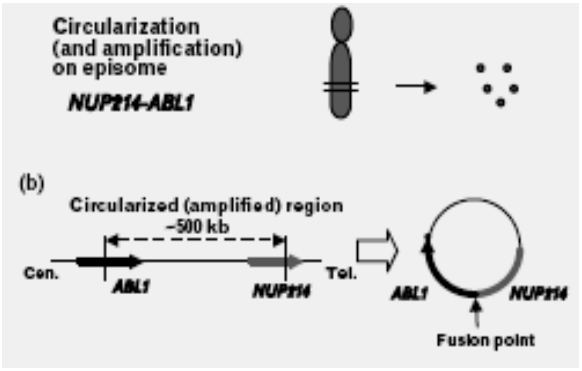


Figure 4 Mechanism of formation of *NUP214-ABL1.* (a) Circularization on episome shown schematically for an hypothetical gene. (b) Scheme of the amplified region and the structure proposed by Craux *et al.*

Gene fusion, resulting in synthesis of fusion genes encoding for chimearic proteins, in most cases generates abberant transcription factors. However, the first documented example of such an abberant fusion gene was described as an abnormal small chromosome 22 in the cells of chromic myelogenous leukaemia (CML), subsequently called the Philadelphia (Ph) chromosome (Nowell, Hungerford, 1960). In the Ph chromosome, the translocation between chromosomes 9 and 22 leads to the fusion of *c-ABL* to the *BCR* gene, the chimaeric protein encoded by the BCR-ABL fusion gene is a tyrosine kinase (Epner, Koeffler, 1990) which activates a number of cell cycle-controlling proteins and enzymes, speeding up cell division. This is an early genetic event which is an important contributor to the development of the CML.
Some examples of genes often associated with cancer and commonly affected by deletions, translocations and inversions (here grouped together as chromosomal translocations) will be shortly described below.

3.3 Deletions

Tumor suppressor genes are negative regulators of cellular proliferation, their inactivation, in most cases through deletions results in loss of the “brake” on tumor growth. To date, numerous tumor suppressor genes have been identified, 174 of them are listed in the tumor suppressor gene database (TSGDB), which contains updated information about putative tumor suppressor genes from human beings and other organisms. This number however most likely will increase in the future. Different tumor suppressor genes have been identified in different spectrum of human cancers, with the probably most prominent retinoblastoma (*RB*) and p53 genes encoding for pRb and p53 proteins. *RB* was the first gene proposed to have a tumor-suppressor function (Comings 1973). The second tumor-suppressor gene p53 was described in 1979, but originally believed to have an oncogene function. Further characterization of this gene in the following 10 year showed however the tumor-suppresive role of p53.
Mutations in the p53 protein are observed in more than 50% of human cancers (Hollstein et al., 1991), there are however different mechanisms how this protein contributes to tumorigenesis, as the loss of

p53 can facilitate resistance to apoptosis, angiogenesis or can lead to genomic instability in cells. As a key component of the DNA damage sensor cascade, inactivation of p53 can prevent the induction of the apoptotic effector cascade (Harris, 1996). On the other hand, p53 positively regulates thrombospondin-1, an inhibitor of angiogenesis. Consequently, the loss of p53 causes low levels of thrombosponin-1, which then leads to release of epithelial cells (Dameron et al., 1994). Furthermore, p53 is considered as stability gene, thus the loss of p53 leads to aneuploidy of cultured cells (Harvey et. al., 1993). Furthermore, p53 is one of the cell-cycle checkpoint genes mostly via stimulation of p21, an inhibitor of cyclin-dependent kinases (CDKs). Through its negative effects on various CDKs, p21 inhibits both the G1/S and the G2/M transitions. Failure of the cell-cycle checkpoint results in loss of the quality-control system which coordinates sequential events within the cell cycle. Thus, DNA damage during either G1 or G2 phase of the cell cycle triggers a cell-cycle arrest (which requires the p53 tumor-suppressor gene) to repair the genotoxic damage or to block further cell proliferation if the damage is irreparable (Levine, 1997). As most human cancers are dependent on sustained p53 inactivation for tumour maintenance (oncogene-addiction), restoring of p53 function has been proposed as new therapeutical approaches, supported by recent study showing tumor regression in vivo after restoration of p53 (Ventura et al., 2007).

3.4 Chromosomal translocations

Chromosomal translocation is an event commonly found in cancer cells, generating gene activation and gene fusion, as mentioned above. Yet, gene fusion, resulting in the synthesis of chimaeric proteins, appears to be the main cause of abnormalities involved in cancer development. Gene fusions have been detected in numerous cancer types of both, haematopoietic and solid tumors. It is a common event leading to formation of tumors; however, the mechanisms how gene fusions contribute to tumorigenesis are greatly different. One of them, activation of a proto-oncogene as a result of chromosomal translocation or inversion was already mentioned.

However, in most cases chromosomal translocations lead to activation of genes or gene fusions encoding for aberrant transcription factors as the consequence. Transcription factors regulate many pathways associated with cell viability like cell growth, differentiation or apoptosis by binding to specific DNA sequences within the promoter regions of corresponding genes and modulating the expression of these genes. Binding of a transcription factor to the specific promoter region can control the switch from inactive to active chromatin, stabilize the transcription complex (which includes RNA polymerase II and initiation factors) on the promoter DNA. Moreover, recruitment of other factors to the complex is another important function of a transcription factor. In most cases such aberrant transcription factors consist of an activation domain linked to a DNA-binding domain or to domains that interact with proteins which then bind DNA (Sánchez-García, Rabbits, 1994).

Numerous abnormal transcription factors are strongly associated with formation and progression of distinct cancer types. Thus, some of the most important members of transcription factor families and the associated cancer types will be described in a separate section.

3.5 Most important transcription factor - families in cancer

The simple model describing the binding of a transcription factor (TF) to the promoter region of its target gene is complicated by the fact that mammalian TFs are often members of large protein families that bind to similar DNA sequences. There are three most prominent TF-families associated with deregulated activity in cancer, namely the Ets, E2F and Myc family.

3.5.1 The Ets transcription factor family

Members of the Ets family play important roles in organogenesis, hematopoiesis, B-cell development and signal transduction (Dittmer, Nordheim, 1998). The Ets DNA binding domain is composed of 85 amino acids forming a helix-turn-helix structure. Some members of Ets family, which are often found as fusion proteins with another protein in various leukemias and as a rare event also in solid tumors, are summarized in Table 1.

Table 1. Ets transcription family members and associated cancer type

TF- family	Affected fusion genes	Associated cancer type
Ets	TEL AML1	ALL
	ABL TEL	AML
	PDGF-β TEL	CMML
	AML1 EAP	MDS
	EWS/FLI1 EWS/ERG	Ewing's sarcoma

ALL,acute lymphoblastic leukemia
AML, acute myelogenous leukemia
CMML, chronic myelomonocytic leukaemia
MDS, myelodysplastic syndrome

It is likely that inappropriate regulation of genes containing Ets binding sites in their promoter leads to the transformation of cells bearing a fusion protein containing an Ets member. The mechanisms of transformation by fusion proteins differ however in distinct cancer types. In ALL, which in about 25% is associated with TEL/AML1 fusion protein, deregulation of AML1 target genes is thought to cause the neoplastic transformation. It appears that the fusion protein downregulates all AML-regulated genes and that the loss of AML-activated gene expression leads to leukemia development. In the case of

EWS/FLI1 fusion protein in Ewing's sarcoma, inappropriate activation of FLI1 targets leads to transformation of cells.

3.5.2 The E2F transcription factor family

The E2F family of transcription factors consists of eight members (E2F1, E2F2, E2F3, E2F4, E2F5, E2F6, E2F7 and E2F8) that function as heterodimers and bind to a consensus sequence known as E2F site in the promoter region of the target genes. All E2F members contain a DNA binding and a dimerization domain and a C terminal transactivation domain (with the exception of E2F6, which does not have a C terminal transactivation domain). A subset of E2F family members, E2F1, E2F2 and E2F3 also contain N-terminal protein interaction domain. Moreover, within the transactivation domain of E2F proteins is a protein interaction domain that enables the binding to Rb family of proteins. E2F1-E2F6 members of E2F family bind to DNA as heterodimers with DP (DP1-4, TFDP1-4: transcription factor Dp-1-4; E2F dimerization partner1-4) family proteins, while E2F7 and E2F8 bind to DNA independent of DP proteins. An overview of E2F transcription family members is shown in Figure 5.

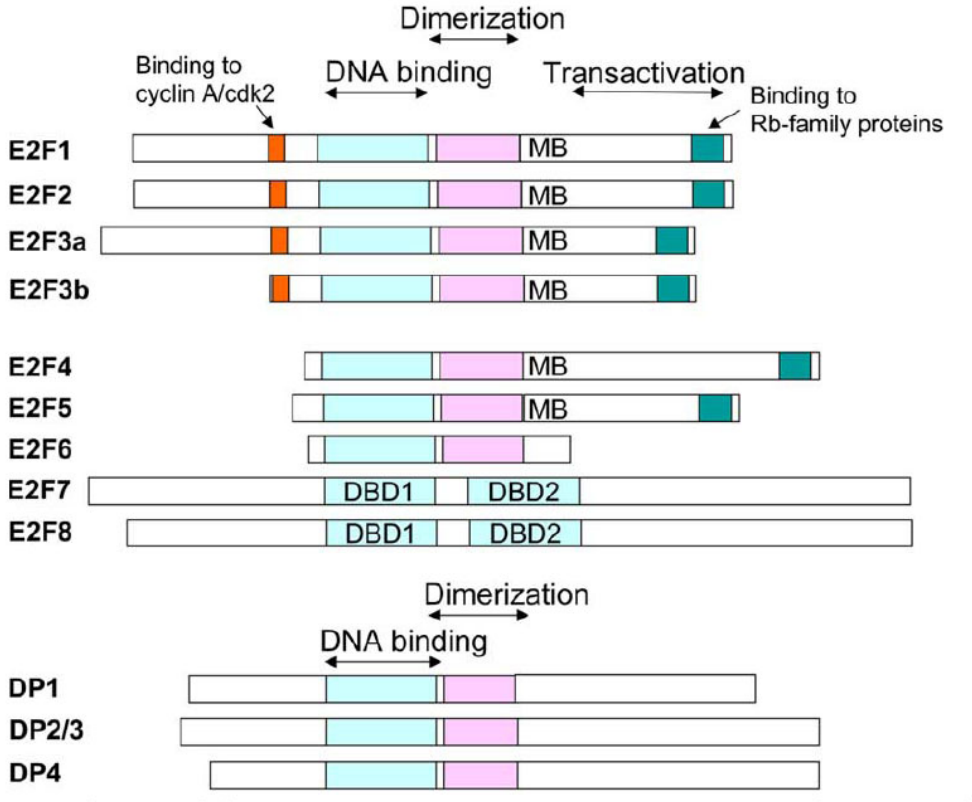


Figure 5 E2F transcription factor family members. The DNA binding domain is indicated in green, the heptad repeat domain required for dimerization is shown in pink, interaction sites with cyclinA/cdk2 or Rb-family proteins are indicated in orange and blue, respectively (DeGregori, Johnson, 2006)

As with the previous members of transcription factor families, E2F family members can both activate or repress transcription of its target genes. The transcriptional activity of E2F1 through E2F5 is regulated through their association with RB or the related pocket proteins, p107 and p130. E2F1, E2F2, and E2F3a are so-called “activators” because they transcriptionally activate E2F target genes such as *cyclin E.* E2F3b, E2F4, and E2F5 are referred as "repressor" E2Fs because they repress E2F target genes. E2F regulates genes whose products are required for DNA synthesis and G1/S or G2/M phase progression, thus leading to stimulation or inhibition of cell proliferation. Additionally, E2Fs control the expression of genes involved in apoptosis, DNA repair, differentiation and development.

3.5.3 The Myc transcription factor family

Members of the Myc family share a carboxyl terminal basic region containing the helix-loop-helix (HLH) (Murreet al., 1989) and leucine zipper (LZ) (Landschulz et al., 1988) motif that mediates sequence-specific DNA binding and dimerization and a transactivation domain at the amino terminus. There are three mammalian myc genes, the c-myc, N-myc and L-myc. All members of the Myc family form a heterodimer with Max, a small, ubiquitously expressed protein that can bind to HLH-LZ protein, directly binds to the DNA (Baudino et al., 2001) and thus mediates the DNA binding of Myc. The transformation property of Myc family members is thought to be mediated through interaction between Myc and other cellular proteins. For example, N-myc can act as transcriptional activator when bound to the promoter region as N-myc/Max heterodimer, and transcriptional repressor when bound as a heterodimer with Mnt, Mxi or Mad (Grandori et al., 2000; Sakamuro et al., 1999). Some important interaction partners of Myc family members are shown in Figure 6.

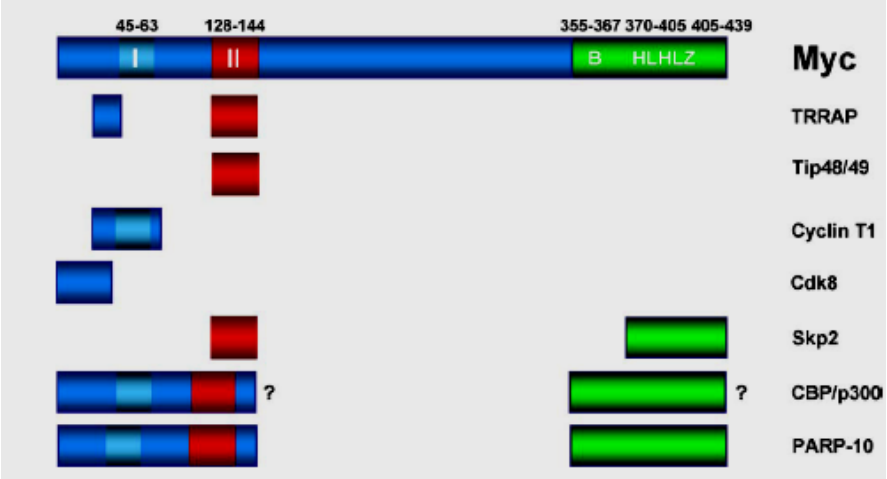


Figure 6 Localisation of binding sites on the Myc transactivation domain for interaction with protein cofactors (Cowling, Cole, 2006)

The oncogenic potential of myc genes can be activated by chromosomal translocations, retroviral insertion or gene amplification which then results in deregulation of cell proliferation, cell cycle

regulation, apoptosis and genomic instability. Consequently, overexpression of Myc family members can also lead to neoplastic transformation (Facchini, Penn, 1998). Deregulation of different Myc family members has been shown to be clearly associated with different types of tumors; N-Myc is amplified in 25% of neuroblastoma and is the best clinical prognostical marker for poor survival (Schwab, 2004). L-Myc amplification is associated with approximately one fourth of primary small cell lung cancers (SCLC) (Makela et al., 1992) and c-Myc is associated with various human tumors. The relative importance of Myc as transcriptional regulator mediating neoplastic transformation remains controversial. The main problem is the difficulty in clearly defining a true Myc target gene. There are various proteins targeting the so-called E-box, which is also the binding region of Myc/Max heterodimers. Therefore, it is difficult to identify specific promoter regions, which are regulated specifically by Myc. Furthermore, Myc has a relatively weak transactivation domain, thus again complicating the identification of target genes. For example, dozens of genes have been proposed to be target genes of c-Myc. Genes suggested to be activated by c-Myc, among them *cad, cdc25a, odc, eIF-4E* and *ISGF3*γ were proposed mainly using subtractive hybridization, differential display, or educated guessing. Nonetheless, the majority of these putative c-Myc target genes has been shown to remain unchanged in c-Myc null cells (Bush et al., 1998). Thus, it is likely that the interaction with other co-factors is essential for mediation of Myc's oncogenic properties.

The identification of transcription factor targets has made a big progress after decoding of the human genome, which provided a huge amount of sequence information, enabling genetic information analysis on a global scale. Therefore, a short historical overview of microarray technology and its application for target gene identification is given below.

3.6 Microarray technology

Microarray technology began to be used to study gene expression profiles in 1990s and to date, various DNA microarray and DNA chip devices and systems have been developed. The very first simple DNA arrays, so-called "dot-blots" have now progressed to the level of high-density microarrays (Southern, 2001).

Nowadays, the term "microarray" most often describes chips or slides containing thousands of DNA elements that are used for simultaneous genome-wide measurement of mRNA levels in order to generate the so-called "gene expression profile".

There are different platforms of microarrays, one of them is the cDNA microarray. The spotted cDNA microarray consists of small aliquots of distinct cloned gene sequences. Each spot contains few hundred bases of a previously characterized sequence known from a cDNA library and represents one expressed gene. There are thousands of such spots on a glass slide. As the levels of mRNA in a particular cell reflect the metabolic activity of the related gene, the strength of the signal intensity measured by cDNA array reflects the amount of the presence of mRNA and thus the relative gene activity in the investigated sample. However, cDNA microarrays are limited by the problem of cross hybridization between mRNAs and other non-specific elements of the cDNA clone.

Another platform of microarrays are oligonuctleotide microarrays, where oligonucleotides (60-mers in Agilent arrays, 25-mers in Affymetrix arrays) are placed on a slide. Two types of oligonucleotides are used, those which match a target sequence exactly are called Perfect Match (PM) and those which have a single mismatch base in the middle of the sequence are called Mismatch (MM). The template, in case of Affymetrix labelled cRNA, binds to these probe pairs, usually 16-20 oligonucleotides with the sequences from different parts of each gene, which constitute the "probe set". The intensity information from the values of each of the probes in a probe set is then combined to obtain an average expression value (Bolstad et al., 2003).

Additionally, protein microarrays using antibodies or tissue microarrays consisting of dozens to hundreds of predefined microscopic elements of tissue arranged on a glass slide are used.

Analysis of microarray data requires multiple steps, including data quantification, a step where translation of signal intensities obtained from microarrays into numerical values takes place. The next step is data normalization, a step to correct the untrue data resulting from differences in experimental procedures such as labelling, hybridisation and washes, diversities between chips and variation in detection procedures. Finally, data mining and modelling are the most important steps to achieve accurate and significant biological conclusion from obtained data.

Taken together, microarray data gives information on the expression level of each individual gene represented on the microarray. Therefore, microarrays have had a significant impact in our understanding of normal and abnormal cell biochemistry, an important aspect in oncology. Microarray approaches resulting in distinct expression profiles for each cancer type have been used for identification of molecularly distinct subtypes of cancers that were viewed previously as homogenous diagnostic categories. Thus, novel insights into molecular taxonomy of many cancers could be revealed. Moreover, a comprehensive investigation of whole pathways and interacting genes could be performed. As a result, gene expression signatures associated with activation of oncogenic pathways in tumors (Huang et al., 2003) or physiological targets of retinoblastoma protein pathway, deregulation of which is a hallmark of human cancer (Vernell et al., 2003) could now be identified using whole-genome microarray analysis. Furthermore, targeted genetic manipulations (Lamb et al., 2003) or cellular responses to physiological stimuli in case of breast cancer (Chang et al., 2005) could be analysed using microarray technology. Microarray analysis of genome-wide expression pattern in tumors promises to improve the diagnostics, risk stratification and therapy outcome prediction for patients. Microarrays can be used to identify novel targets of oncogenic transcription factors, whose modulation might inhibit or reverse disease progression. By the use of tissue or cell microarrays also validation of the found targets can be performed.

3.7 Identification of oncogenic transcription factor targets

Identification of transcription factor targets is required to understand by which pathways the oncogenic property of many transcription factors are mediated. Target gene specificity is partly determined by the DNA binding domain of transcription factors, but also the promoter context, as for example the relation of the DNA binding site in relation to other promoter elements, contributes to the gene-specific control through transcription factors.

Different methods have been applied in order to determine regulatory DNA binding motifs, like monitoring of gene expression using Nothern blot or RNAse protection assay, furthermore mutations of promoter sequences (promoter bashing), cDNA filter arrays and other “hit-or-miss” approaches were performed. These methods however were all limited to the study of previously identified genes and only quite a low number of genes can be monitored.

Development of microarray technology has been a big progression for identification of transcription factor targets. Using the microarray approach, all genes which exhibit significant changes upon inactivation of a transcription factor can now be identified. Thus, all the genes present in the whole genome can be monitored by this method simultaneously. However, identification of genes which represent direct targets of a transcription factor is also dependent on the number and nature of indirect effects. Thus, the microarray expression profiling on its own is not sufficient for identification of direct transcription factor targets and additional methods are needed for verification of putative target genes. Commonly used methods for confirmation of microarray data are usually RT-PCR and immunohistochemistry. For identification of direct targets the combination of microarray technology with ChIP can be used. ChIP technique uses antibodies against specific transcription factors to isolate bound DNA sequences embedded in chromatin (Orlando et al., 1997).

4 Paediatric cancer

In adults, genetic events leading to cancer are generally not inherited through the gametes; rather, they are changes in the DNA of somatic cells. For most solid human tumors, 15-20 years from carcinogen exposure to clinical detection of a tumor is needed. The major risk factors for cancer are tobacco, alcohol, diet, sexual and reproductive behavior, infectious agents, family history, occupation, environment and pollution. Whereas cancer has a very common incidence in adults (worldwide an estimated 11 million cancer cases were diagnosed in 2002, one quarter being in Europe), cancer is very rare event among children everywhere in the world. In industrialized countries, only about 0.5% of all cancers occur among children aged less than 15 years. Obviously, the mechanisms of cancer development must be different for children, indicated by age when cancer is detected and different types of cancer.

Most cancer types occurring in children are already arising during embryonic development through acquired intrauterine genetic abnormality or an inherited genetic predisposition. Whereas most adult cancers are carcinomas, childhood cancers are histologically very diverse. The 12 major groups are leukaemias, lymphomas, brain and spinal tumors, sympathetic nervous system tumors, retinoblastoma, kidney tumors, liver tumors, bone tumors, soft-tissue sarcomas, gonadal and germ-cell tumors, epithelial tumors, and other and unspecified malignant neoplasms. The following sections will be restricted to the subgroup of sarcomas.

4.1 Sarcoma

Malignant neoplasms that arise from cells of mesenchymal origin (for example, bone muscle, cartilage and other connective tissues) are called sarcomas. Sarcomas are a diverse group of relatively rare malignancies. Soft tissue sarcoma is a cancer type which occurs ten times more frequent in children than in adults. Correct determination of histological subtype of sarcoma is very important for exact diagnosis of sarcoma and subsequent treatment. Development of new diagnostic techniques like quantitative real-time PCR or microarray analysis improved the accuracy and gave new insights into molecular properties of sarcomas. Genetically, sarcomas fall into two main categories. One group of sarcomas is characterized by a tumour-specific translocation that seems to be central to the pathogenesis of the tumour, and is used as diagnostic criteria. Another group of sarcomas are characterized not by a tumour-specific genetic alteration but by complex karyotypes that are characteristic of severe genetic and chromosomal instability. To date, a subset of sarcomas comprised of fourteen sarcoma types has been identified which is associated with specific translocations generating novel fusion oncoproteins (Xia, Barr, 2005). Some of these translocations are listed in Table 3.

Table 3. Chromosomal translocations in sarcomas.

Sarcoma type	Translocation	Fusion product
Alveolar rhabdomyosarma	t(2;13)(q35;q14) t(1;13)(p36;q14)	PAX3/FKHR PAX7/FKHR
Ewing's sarcoma	t(11;22)(q24;q12) t(21;22)(q22;q12)	EWS/FLI1 EWS/ERG
Clear-cell sarcoma	t(12;22)(q13;q12)	EWS/ATF1
Desmoplastic small-round cell tumor	t(11;22)(p13;q12)	EWS/WT1
Muxoid liposarcoma	t(12;16)(q13;p11) t(12;22)(q13;q12)	FUS/CHOP EWS/CHOP

Table adapted from Xia, Barr, 2005

The oncogenic character of these chimeric fusion proteins was shown in different studies, where transformation of primary cells was achieved by overexpression of EWS/FLI1 in immortalised mouse NIH3T3 cells (May et al., 1996), PAX3/FKHR in chicken embryo fibroblast (Scheidler et al., 1996;), NIH3T3 cells (Lam et al., 1999) and in human embryonal RMS (eRMS) cell line (Anderson et al., 2001) or FUS/CHOP in NIH3T3 cells (Schwarzbach et al., 2004). Tumorigenic function of these transcription factors was also further supported in vivo by the use of xenografts in mice for EWS/FLI1 (Arvand et al., 2001), for FUS/CHOP (Perez-Mancera et al., 2002) and for PAX3/FKHR (Keller et al., 2004). However, the tumor formation frequency was fairly low in these experiments and could be increased by combination with additional genetic alterations. Therefore, it is likely that expression of a fusion gene alone is not sufficient for tumor formation and that in most cases an additional collaborating genetic alterations are required.

4.2 Transcription factors and their target genes

Not only sarcomas are associated with specific translocations leading to formation of aberrant transcription factors. Therefore, three examples of chromosomal translocations leading to fusion of two genes encoding for transcription factors and their targets are summarized in this section. First, two members of Ets transcription factor family, AML1 and AML1- consisting fusion genes and EWS-Ets (see also Table 1) will be described. Second, the transcription factor associated with alveolar Rhabdomyosarcoma, PAX3/FKHR, will be shortly discussed.

The TEL/AML1 fusion gene occurs in childhood B-cell acute lymphoblastic leukemia (ALL) as a result of the translocation of human chromosome 12;21. TEL is a member of Ets family, but in TEL/AML1 the N-terminal helix-loop-helix domain of TEL is fused to a nearly complete AML1 gene, thus leading to deregulation of AML1 target genes. Many of AML1 targets are mostly genes which are important for normal differentiation of hematopoietic progenitor cells and which regulate cell proliferation and survival. AML1 binds to the DNA sequence TGTGGT, the TEL/AML1 fusion leads to inhibition of basal

promoter activity, thus leading to repression of AML1 target genes. Some of AML1 and AML1-containing fusion gene targets are listed in Table 4.

Table 4. Target genes of AML1 and AML1- consisting fusion genes

Gene	Target	Reference
AML1	GM-CSF	Frank et al., 1995
	IL-3	Uchida et al., 1999
	M-CSF	Zhang et al., 1996
	Myeloperoxidase	Britos-Bray and Friedman, 1997
	Neutrophil elastase	Nuchprayoon et al., 1994
	Granzyme B	Babichuk and Bleackley, 1997
	NP-3	Westendorf et al., 1998
	TCRβ	Prosser et al., 1992
	TCRγ	Hsiang et al., 1993
	TCRδ	Redondo et al., 1992
TEL/AML1	TCRβ	Hiebert et al., 1996
	IL3	Uchida et al., 1999
	M-CSF	Fears et al., 1993
AML1/ETO	GM-CSF	Frank et al., 1995
	IL-3	Mao et al., 1999
	NP-3	Westendorf et al., 1998

EWS/FLI1 is a common fusion gene associated with Ewing's sarcoma, consisting of the potent transactivation domain of EWS and the DNA-binding domain of FLI1, a member of Ets transcription family. Over the past several years numerous studies were published about the targets of *EWS/FLI1* transcriptional activation using representational difference analysis, microarray analysis or chromatin immunoprecipitation, thereby identifying plenty of putative EWS/ETS target genes. However, these target genes were mainly identified by transfection of fusion gene into different cell lines followed by various subtraction techniques mentioned above to isolate differentially expressed genes. The overexpression of EWS-ETS fusion protein in cell lines that don't derive from Ewing tumors or the use of a very low number of Ewing cell lines led often to results which were then not statistically significant. Moreover, only few of the potential target genes identified in microarray screen have been validated by independent methods. Therefore, only some of the target genes confirmed as directly regulated by EWS-ETS fusion proteins in Ewings sarcoma are listed in Table 5.

Table 5. Target genes of EWS-Ets family

Genes	Targets	Reference
EWS-ETS:	TGFBR2	Hahm et al., 1999
	Phospholipase C-beta	Dohjima et al., 2000
EWS-FLI1 } Group1	Uridine Phosphorylase	Deneen et al., 2003
EWS-ERG } Group1	c-myc	Nishimori et al., 2002
EWS-FEV } Group1	ID-2	May et al., 1997
	PDGF-C	Zwerner and May, 2001
	PIM-3	Deneen et al., 2003
EWS- ETV1 } Group 2	TNC	Watanabe et a., 2003
EWS-E1AF } Group 2	NKX2.2	Owen and Lessnick, 2006
	CAV-1	Tirado et al., 2006
	DAX-1	Mendiola et al., 2006

PAX3/FKHR is not a member of the ETS transcription factor family. However, also in this case, two transcription factors, PAX3 and FKHR are fused by a translocation leading to formation of a more potents transcription factor. PAX3/FKHR DNA–binding domain derives from PAX3 and contains a paired domain and additional octapeptide and homeodomain. PAX3/FKHR is - similar to EWS-ETS transcription factors - associated with a particular sarcoma type, namely alveolar rhabdomyosarcoma. The oncogenic potential of PAX3/FKHR is well documented; however, the pathways and downstream targets of PAX3/FKHR are still not completely characterized. The target genes of PAX3 and PAX3/FKHR can be divided in three groups: there are genes which are regulated by both Pax3/FKHR and Pax3, there are targets induced by Pax3/FKHR but not Pax3, such as PDGFαR and finally genes induced by Pax3/FKHR but repressed by Pax3. Some target genes are listed in Table 6.

Table 6. Target genes of PAX3/FKHR

Genes	**Targets**	**References**
PAX3	NCAM	Edelman et al., 1998
PAX3	MyoD	Maroto et al., 1997
PAX3	c-met	Epstein et al., 1996; Ginsberg et al., 1998
PAX3	MYCN	Khan et al., 1999
PAX3/FKHR	Bcl-xl	Marque et al., 2000
PAX3/FKHR	CB1	Begum et al., 2005
PAX3/FKHR	BMP4	Begum et al., 2005
PAX3/FKHR	MBP	Kioussi et al., 1995
PAX3/FKHR	PDGFαR	Epstein et al., 1998
PAX3/FKHR	GLUT4	Armoni et al., 2002

4.3 Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children, accounting for about 8% of all cancers occurring in children (Pappo et al., 1999). RMS often develops in the head and neck region of the body, but also other cases with the appearance of RMS at any site in the body are known, see also the indicated locations in the anatomical picture in Figure 7.

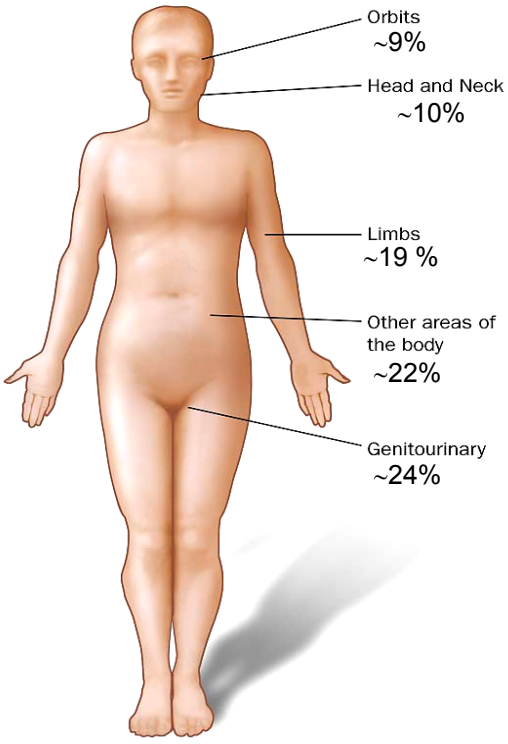


Figure 7 Anatomic localization sites of RMS. Common RMS sites are indicated in percentages (Pappo et al., 1995)

Four histologically different subtypes of RMS are known, the embryonal, botroidal, alveolar and pleomorphic RMS with the common embryonal and alveolar subtypes accounting together for approximately 80% of all cases.

Embryonal Rhabdomyosarcomas (eRMS) are the most common type of rhabdomyosarcoma, and it typically occurs in children at the age of less then 10 years. There is no specific genetic alteration observed in eRMS, but eRMS is often associated with loss of heterozygosity of 11p15. However, typically there are several alterations visible on a karyogramm derived from eRMS cells, as shown in Figure 7B.

Botryoid Rhabdomyosarcoma often occurs in hollow organs, such as the bladder, vagina and uterus. It's often seen in infants and toddlers and accounts for about 6% of all RMS.

Alveolar Rhabdomyosarcomas (aRMS) typically affects older children and teenagers. Malignant cells normally affect large muscles forming hollow spaces (alveoli). Most of the cases of aRMS are associated with a specific translocation t(2;13) or (1;13). Genetic consequences of this event will be described more precisely in following section.

Pleomorphic rhabdomyosarcoma can be found in extremities or the trunk. In contrast to other RMS subtypes it mostly occurs in adults and not in children.

Typical cytogenetic changes of two main subtypes, embryonal and alveolar rhabdomyosarcoma, represented by respective karyogram are shown in Figure 8. Genetic alterations involved in tumorigenesis of these two subtypes will be discussed in more detail in following section.

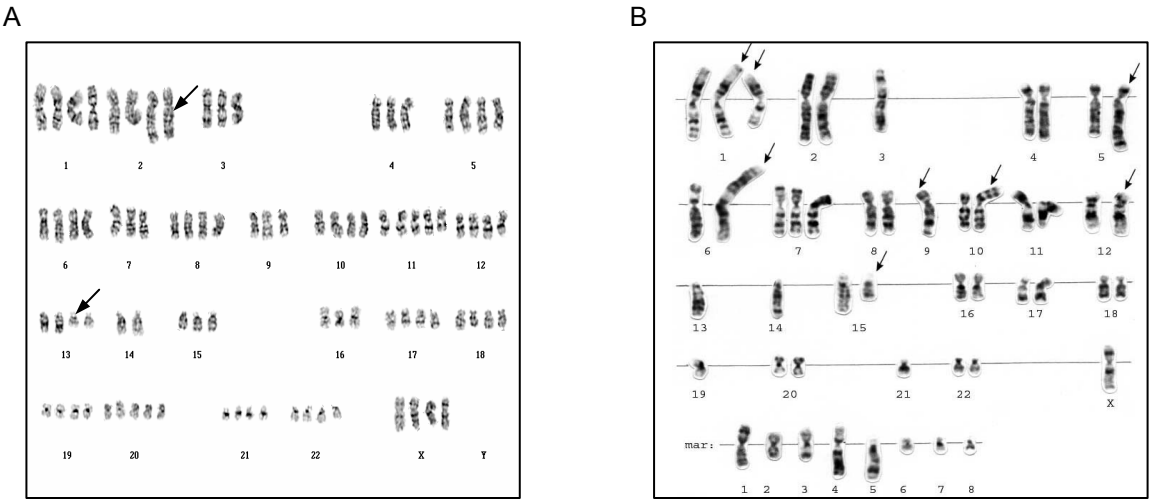


Figure 8 Typical karyogram shown for aRMS (A) and eRMS (B). In aRMS, a typical translocation t(2;13) is indicated. In eRMS, a complex karyotype bearing different alterations are shown (www.htcl.cytspb.rssi.ru/tomors/RD)

4.4 PAX genes

The PAX gene family comprises nine family members, namely PAX1 to PAX9. All members of the PAX genes family contain an amino-terminal 128 amino-acid DNA-binding domain, termed the paired box domain. The paired box domain also gave the name for the family. Moreover, PAX3 and PAX7 also contain a 60 amino-acid DNA-binding homeodomain and octapeptide motif. The homeodomain is also known to play important roles in developmental processes of many organisms. While certainly not the only developmental control genes, they have been shown to play crucial roles from the earliest steps in embryogenesis to the very latest steps in cell differentiation. The domain binds DNA through a helix-turn-helix (HTH) structure. The HTH motif is characterised by two alpha-helices, which make intimate contacts with the DNA and are joined by a short turn. The second helix binds to DNA via a number of hydrogen bonds and hydrophobic interactions, which occur between specific side chains

and the exposed bases and thymine methyl groups within the major groove of the DNA (Schofield, 1987).

PAX genes play a role in embryonic tissue development and cellular differentiation through promotion of cell proliferation, cell-lineage specification, migration and survival (Robson, He and Eccles, 2006). Due to this important role of PAX genes in development they also can contribute to cancer development if dysregulated. However, the precise role of PAX transcription factors in cancer is not known. Fife members of the PAX gene family - PAX2, PAX3, PAX5, PAX7 and PAX8 - are thought to have a tumour promoting function. Furthermore, PAX genes are often overexpressed in a wide range of cancer cell lines (Muratovska et al., 2003). The processes that are involved in tumorigenesis – limitless replicative potential, self-sufficiency in growth signals, resistance to apoptosis and gain of invasive and metastatic potential - can be misregulated by these genes in different manners, as summarized in Figure 9.

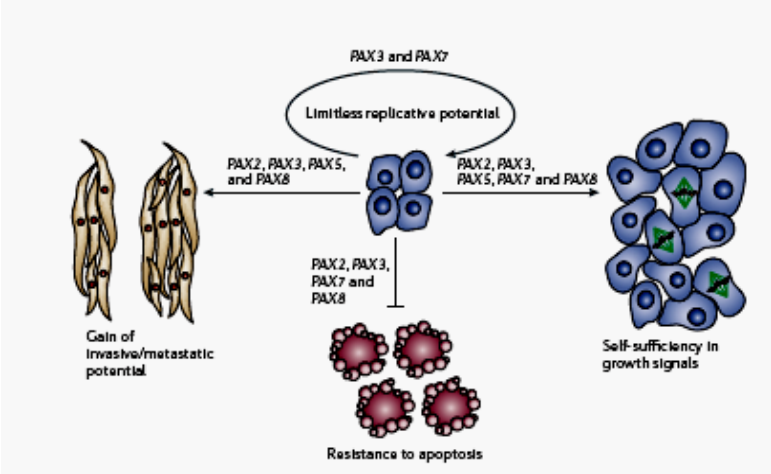


Figure 9 Cancer contributing characteristics regulated by PAX genes that are favourable for tumor progression (Robson, He, Eccles, 2006)

For development of RMS, PAX3 and PAX7 gene expression plays an important role. Tumor-associated PAX3 expression has been detected in eRMS. Moreover, aRMS in most cases is associated with expression of an abberant transcription factor generated by fusion of two genes PAX3 or PAX7 and FKHR, also known as FOXO1A (Galili et al., 1993). The formation of PAX3/FKHR is schematically shown in Figure 10.

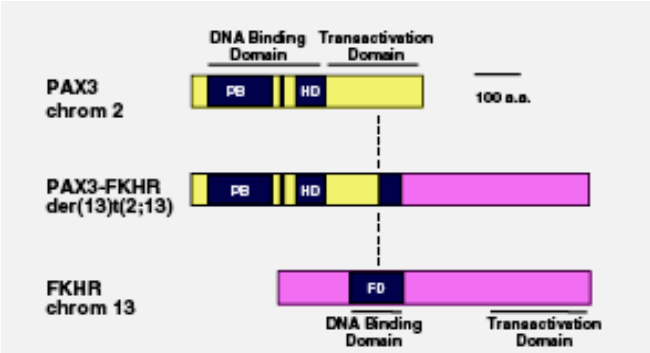


Figure 10 Scheme of the generation of PAX3/FKHR fusion gene. The fusion protein PAX3/FKHR contains the two DNA-binding domains of PAX3 and the transactivation domain derived from FKHR (Xia, Barr, 2005)

On the molecular level, PAX3/FKHR is a stronger transactivator compared to wild-type PAX3 (Bennicelli et al., 1995; Fredericks et al., 1995). Therefore, the oncogenic properties of PAX3/FKHR are thought to base on dysregulation (i.e. up-, or downregulation) of PAX3 target genes. The identification of PAX3/FKHR target genes is one of the main goals of research approaches of this work.

5 Subject of investigation

Taken together, studying endogenously expressed PAX3 in eRMS and PAX3/FKHR in aRMS cell lines provides the best opportunity to define the function of this fusion in tumor development. For the study of these genes a silencing approach using RNAi was applied. The role and mechanism by which PAX3/FKHR participates in aRMS development will be further analyzed in following studies, as many critical targets remain to be identified. The ultimate goal of these studies is to determine the full complement of PAX3/FKHR target genes, and to determine the mechanisms by which diverse target genes mediate aRMS development. We hope that this approach will identify new therapeutic targets that can be used to treat patients with this devastating disease.

Finally, the approach we describe can be applied to any of a variety of tumor types associated with particular recurrent genetic abnormalities. These would include the translocation-associated sarcomas, such as Ewing's sarcoma, synovial sarcoma, and myxoid liposarcoma, and translocation-associated leukemias, including ALL containing TEL-AML1 rearrangements. By combining experimental approaches in appropriate cell types with validation against clinical samples, we anticipate that the data obtained using this approach will yield clinically-relevant datasets that can be used to identify new therapeutic targets in a variety of diseases.

6 Results

Comparative expression profiling identifies an in vivo target gene signature with TFAP2B as a mediator of the survival function of PAX3/FKHR

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(manuscript accepted)

Comparative expression profiling identifies an in vivo target gene signature with TFAP2B as a mediator of the survival function of PAX3/FKHR

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Running title: PAX3/FKHR target genes

Keywords: alveolar RMS / chimaeric transcription factor / expression profiling/ TFAP2B / PAX3/FKHR

Word count of the manuscript: 5647

Abstract

The chromosomal translocation t(2;13), characteristic for the aggressive childhood cancer alveolar rhabdomyosarcoma (aRMS), generates the chimaeric transcription factor PAX3/FKHR with a well known oncogenic role. However, the molecular mechanisms mediating essential pathophysiological functions remain poorly defined. Here, we used comparative expression profiling of PAX3/FKHR silencing in vitro and PAX3/FKHR specific gene signatures in vivo to identify physiologically important target genes. Hereby, 51 activated genes, both novel and known, were identified. We also found repression of skeletal muscle-specific genes suggesting that PAX3/FKHR blocks further differentiation of aRMS cells. Importantly, TFAP2B was validated as direct target gene mediating the anti-apoptotic function of PAX3/FKHR. Hence, we developed a pathophysiologically relevant transcriptional profile of PAX3/FKHR and identified a critical target gene for aRMS development.

Introduction

Rhabdomyosarcoma (RMS) is a common childhood soft tissue sarcoma associated with the skeletal muscle lineage. Based on histology, two main subgroups of RMS, embryonal (eRMS) and alveolar (aRMS), are distinguished. The majority of cases of the more aggressive aRMS are associated with one of two reciprocal translocations t(2;13)(q35;q14) or t(1;13)(p36;q14), generating intronic fusions of PAX3 (or PAX7) and FKHR, also known as FOXO1A (Galili *et al.*, 1993). The fusion proteins contain the two DNA-binding domains of PAX3 or PAX7, namely a paired– and a homeo-domain and the transactivation domain derived from FKHR.

Earlier studies on PAX3/FKHR support an oncogenic role of this fusion protein in tumor initiation and maintenance. A tumor initiating effect has been evaluated in vitro where ectopic expression of PAX3/FKHR leads to transformation of chicken embryo fibroblast (Scheidler *et al.*, 1996), NIH3T3 cells (Lam *et al.*, 1999) and a human embryonal RMS (eRMS) cell line (Anderson *et al.*, 2001). Furthermore, PAX3/FKHR induces murine aRMS postnatally in cooperation with other oncogenic events such as loss of Ink4A/ARF (Keller *et al.*, 2004). Involvement in tumor maintenance is reflected by the fact that downregulation of fusion protein activity by antisense oligonucleotides induces apoptosis (Bernasconi *et al.*, 1996). Similarly, an inducible transcriptional repressor induced tumor regression in vivo via extensive apoptosis (Ayyanathan *et al.*, 2000) suggesting that established RMS tumors are dependent on PAX3/FKHR expression.

On the molecular level, PAX3/FKHR is a stronger transactivator compared to wild-type PAX3 (Bennicelli *et al.*, 1995; Fredericks *et al.*, 1995). Therefore, the oncogenic properties of PAX3/FKHR are thought to base on dysregulation (i.e. up-, or downregulation) of PAX3 target genes. However, recent studies suggested that PAX3/FKHR might alter the expression of gene targets quantitatively and qualitatively distinct from PAX3 (Begum *et al.*, 2005; Epstein *et al.*, 1998). Understanding the oncogenic function of PAX3/FKHR hence requires identification of the pathophysiologically relevant target genes. One difficulty in this search has been that most studies relied on heterologous cell systems to ectopically express PAX3/FKHR. Furthermore, most of these studies used clones stably expressing the fusion protein which precludes discrimination of direct from indirect regulatory events. Nevertheless, a number of potential target genes have been suggested by studies performed in heterologous systems such as c-met (Epstein *et al.*, 1996; Ginsberg *et al.*, 1998), MYCN (Khan *et al.*, 1998), bcl-xl (Margue *et al.*, 2000), CNR1 and BMP4 (Begum *et al.*, 2005) or CXCR4 (Tomescu *et al.*, 2004). However, the pathophysiological role of these target genes regarding aRMS development or maintenance remains largely unclear.

Therefore, we developed a system to analyze PAX3/FKHR dependent gene expression in aRMS itself. This system consists of two different parts: first, endogenous PAX3/FKHR was downregulated by RNAi (Elbashir *et al.*, 2001) in aRMS cells in culture followed by gene expression profiling. Second, expression signatures specific for PAX3(7)/FKHR-translocations were identified in aRMS tumor biopsies (Wachtel *et al.*, 2004). Comparative expression analysis then was able to identify genes that are dysregulated by PAX3/FKHR both in vitro and in vivo. Functional studies furthermore revealed that one of these direct target genes, TFAP2B, acts as an essential mediator of PAX3/FKHR function in cell survival.

Results

PAX3/FKHR promotes aRMS cell survival

To characterize PAX3/FKHR target genes relevant for aRMS development and maintenance, we established a siRNA-mediated down-regulation strategy. Initially, nine different siRNA molecules against both the PAX3 part and the PAX3/FKHR breakpoint region were tested. siRNAs against the breakpoint region failed to efficiently down-regulate the message (data not shown). The remaining siRNA duplexes resulted in specific down-regulation efficiencies up to 50% of the original mRNA levels as measured by qRT-PCR. Higher silencing efficiencies were subsequently achieved by different combinations, whereby two siRNAs led to the most effective specific inhibition of 70% in RD eRMS cells and by 80% in Rh4 aRMS cells (Figure 1A). On the protein level, a reduction to 50% of PAX3/FKHR in Rh4 cells and to 40% of PAX3 in RD cells was observed compared to control siRNAs (Figure 1B). No unspecific interferon response was observed (data not shown). Therefore, this combination of siRNAs was used in all silencing experiments. As in translocation-positive aRMS cells alleles for both PAX3 and PAX3/FKHR are present, siRNAs targeting the PAX3 part of the fusion protein could down-regulate both PAX3 and PAX3/FKHR. However, we found PAX3 >1000 fold less expressed than PAX3/FKHR in Rh4 cells suggesting that wild-type PAX3 expression can be neglected (data not shown).

To characterize the physiological effects upon siRNA treatments, proliferation of RD and Rh4 cells after 24hrs, 48hrs and 72h of PAX3 silencing was measured. Cell growth was found to be inhibited significantly in cells treated with PAX3 and PAX3/FKHR siRNA, but not in untreated or control treated cells (Figure 1 C, D). Furthermore, active caspase 3/7 showed an approximately 2-fold increase specifically in cells with silenced PAX3 and PAX3/FKHR expression (Figure 1 E, F). Thus, an anti-apoptotic function of PAX3 in eRMS and PAX3/FKHR in aRMS cell lines could clearly be demonstrated, as anticipated from earlier findings (Bernasconi et al., 1996). This validates our siRNA approach on the physiological level.

Comparative microarray analysis reveals novel candidate PAX3/FKHR target genes

Next, we sought to identify candidate target genes of PAX3/FKHR using gene expression profiling after treatment of aRMS cells with siRNA and corresponding controls for 24hrs, 48hrs and 72hrs. As a model system Rh4 cells were chosen based on previous expression profiling data indicating that Rh4 cells represent most closely in vivo biopsies (see supplementary material, Figure 1). The microarray data were analysed using the

GeneSpring 7.0 software (Figure 2 A). Genes downregulated after 24hrs of PAX3/FKHR silencing and therefore representing putative direct PAX3/FKHR targets, were selected. At the shortest time point of treatment (24 hrs) at which cellular apoptosis does not yet play a major role 1834 genes were specifically down-regulated (>1.5-fold) when compared to scRNA treatment (see supplemental material S1). This list of genes identified in vitro was then compared to PAX3(7)/FKHR-translocation specific gene signature of 299 genes identified in aRMS tumor biopsies, representing putative in vivo PAX3/FKHR target genes (Wachtel *et al*., 2004). This comparison finally generated an overlapping list of 51 genes, which is statistically highly significant ($p < 0.001$) and not generated simply by chance (Figure 2B, C). These 51 genes therefore represent a transcriptional profile of in vivo PAX3/FKHR target genes. They were grouped into different functional classes as indicated in Figure 2D and Table 1. The largest set of genes including FGFR2 and CB1 (CNR1) appear to be involved in signal transduction (25%). Confirming our strategy, CB1 has been recently identified as a direct target of PAX3/FKHR (Begum et al., 2005). The second largest number of genes encode proteins with enzymatic activity (20%). Among them are ADAM10 and ADAM19 metalloproteinases which might be involved in the enhanced metastatic capability of aRMS cells. Finally, several genes are involved in transcriptional regulation and DNA-binding such as the POU domain transcription factor POU4F1 and TFAP2B.

Apart from down-regulated genes, we also found a group of genes upregulated 72hrs after PAX3/FKHR silencing suggesting that expression of these genes is normally repressed by PAX3/FKHR. This group was comprised of 260 genes (see supplemental material S2). Upregulation was specifically observed only starting at 48hrs, suggesting an indirect effect of PAX3/FKHR (Figure 3A). Interestingly, genes with most prominent upregulation (up to 58-fold) are all related to normal myogenic differentiation. These included myosin light chain, troponin C, troponin I, crystalline alphaB and skeletal muscle myosin heavy chain. To confirm these findings, the expression values of two upregulated genes, TNNC2 (34.4-fold) and MYL1 (6.1-fold), were validated by qRT-PCR (Figure 3C). Interestingly, upregulation was specific for aRMS cells and not detected after downregulation of PAX3 in eRMS cells (Figure 3 B, D). Therefore, these experiments support the hypothesis that one of the oncogenic functions of PAX3/FKHR is to block terminal differentiation. Furthermore, they suggest that the cellular background has a profound effect on target genes bound and activated by PAX3 and/or that the target gene spectra of PAX3 and PAX3/FKHR differs (Zhang and Wang, 2006) .

TFAP2B is a direct target gene of PAX3/FKHR

One of the oncogenic functions of PAX3/FKHR is promotion of cell survival. Therefore, it was surprising that no classic apoptotic genes could be identified in our system. However, one of the potential target genes, TFAP2B, has previously been implicated in apoptosis in a mouse model (Moser *et al.*, 1997). We therefore further characterized this potential target gene, first by verification of the microarray expression levels after siRNA treatment by qRT-PCR with CB1 as a control (Figure 4A).

Next, we studied the impact of PAX3/FKHR on transcription of these genes in 293T cells, which normally express neither CB1 nor TFAP2B at substantial levels. As expected, after ectopical expression of PAX3/FKHR, transcription of both endogenous genes was induced ~5-fold (Figure 4B). To test whether direct DNA-binding is necessary, we used PAX3/FKHR mutants in which either the paired or the homeodomain DNA-binding domain contains inactivating point mutations (Xia & Barr, 2004). Interestingly, CB1 transcription was not induced by the mutant bearing a non-functional homeodomain, whereas TFAP2B transcription was not induced by the mutant with an impaired paired domain. We therefore conclude that activation of CB1 depends mainly on the PAX3 homeodomain, that of TFAP2B however on the paired domain (Figure 4B). Full transcriptional activation however, can only be reached using the intact PAX3/FKHR protein.

To identify potential paired domain binding sites in the TFAP2B promoter, 3200 bp upstream of the transcriptional start site and deletion constructs thereof were cloned in front of a luciferase reporter and used in reporter assays in 293T cells. PAX3/FKHR induced a significant transactivation of up to 3-fold compared to control with the full-length as well as a shorter 1.5kb (deletion construct 1, TFAP2b_1) reporter construct (Figure 4C). Further deletion down to 0.8kb (deletion construct 2, TFAP2b_2) reduced transactivation significantly. Transactivation of the 1.5kb construct also was dependent on an intact paired domain as observed before (Figure 4D). Therefore, these experiments suggested a potential binding site for the PAX3/FKHR paired domain between -1592 bp and -806 bp. To verify these results we next performed a chromatin immunoprecipitation experiment to test for direct binding of PAX3/FKHR to this promoter region. Deletion constructs 1 and 2 were cotransfected with a his-tagged PAX3 construct into 293T cells and immunoprecipitated with either control anti-IgG or specific anti-his antibodies. We recovered a 2-fold higher amount of TFAP2B promoter DNA from the specific immunoprecipitation of deletion construct 1compared to the control but not of deletion construct 2. These experiments verify that PAX3/FKHR can bind to the TFAP2B promoter in the region -1592 to -806 (Figure 4E). In this region three potential PAX3/FKHR binding sites at positions -1461, -1252 and -1186 were identified (Figure 4F). Using site directed mutagenesis these sites were deleted individually and

tested in a reporter assay. Whereas promoter fragments with deletion of the site -1461 (deletion 1) could be activated >3-fold, deletion of the six nucleotides gttccg at position -1252bp (deletion 2) reduced the transactivation potential of PAX3/FKHR to background levels. As this motif has previously been described as binding motif for paired domains (Mayanil *et al.*, 2001) it represents a likely binding site for the PAX3/FKHR paired domain. Deletion 3 at position -1186 also showed reduced activity (2-fold) suggesting that this site plays an assistant role.

To confirm these data, an electrophoretic mobility shift assay was performed with double-stranded oligonucleotides corresponding to deletion sites 2 and 3. As expected, DNA-protein binding could be observed after ectopical expression of his-tagged PAX3 (Figure 4H) and was comparable when using PAX3/FKHR (data not shown). Furthermore, DNA-protein binding with the oligonucleotide specific for deletion site 3 was considerably weaker, consistent with the observation made with reporter deletion constructs (Figure 4G).

We conclude from these experiments that TFAP2B is a novel direct target gene of PAX3/FKHR whose transactivation is dependent on two DNA-binding motifs recognized by the PAX3 paired domain at positions -1252bp and -1186bp.

TFAP2B mediates antiapoptotic function of PAX3/FKHR

TFAP2B has been shown to suppress *myc*-induced programmed cell death in a range of cell lines (Moser *et al.*, 1997). Therefore, we hypothesized that the pro-survival function of PAX3/FKHR might depend on expression of TFAP2B as a PAX3/FKHR target gene. To test this hypothesis, we first investigated the effects of specific TFAP2B silencing on cell survival. siRNA mediated silencing of TFAP2B resulted in efficient down-regulation of TFAP2B expression on mRNA (80%) as well as on protein (50-60%) level (Figure 5A, B). Down-regulation of TFAP2B resulted in suppression of cell proliferation, not observed in control treated cells. Importantly, specific siRNA treatment also increased the rate of apoptosis as measured by an increase in caspase 3/7 activity by 1.7-fold (Figure 5D). The number of apoptotic cells significantly increased from 3% to 41% (Figure 5E), an increase very similar to the result observed after PAX3/FKHR silencing (3% to 36%). These experiments suggest that the anti-apoptotic function of PAX3/FKHR might be mediated, at least in part, by direct transcriptional activation of TFAP2B.

To test this directly, Rh4 cells stably overexpressing TFAP2B from a heterologous promoter were generated. PAX3/FKHR was down-regulated by siRNA treatment and cell proliferation and apoptosis rate measured as before. In these cells proliferation was rescued to almost normal levels (Figure 6A) and the number of dead cells

increased only slightly (1.6-fold) whereas dead cells increased 5.1-fold in non-transfected and 3.5-fold in mock-transfected Rh4 cells (Figure 6B). We conclude from these experiments that TFAP2B acts downstream of PAX3/FKHR to mediate, at least part, of its anti-apoptotic function and therefore represents an essential target gene of PAX3/FKHR.

Discussion

The identification of physiologically relevant PAX3/FKHR target genes is crucial for understanding the oncogenic function of this chimaeric transcription factor. Analysis of target genes has been hampered by the use of heterologous cell systems to study the fusion protein. Here, we used patient-derived aRMS cells to analyze PAX3/FKHR target genes using a loss-of-function silencing approach, in parallel to data acquired from tumor biopsies. This approach allowed the identification of a large set of bona fide PAX3/FKHR target genes.

The choice of aRMS cells to be used as a model system was important and based on previous expression profiling data. These indicated that Rh4 cells most closely reflect in vivo biopsies and therefore are best suited to serve as model for aRMS tumors (Wachtel *et al.*, 2004). Similar to other aRMS cells (Ayyanathan *et al.*, 2000; Bernasconi *et al.*, 1996) we found that ongoing expression of PAX3/FKHR is required for Rh4 cell survival, suggesting that aRMS cells are "addicted" to PAX3/FKHR expression. Measuring changes in the transcriptome at different time points after silencing of PAX3/FKHR revealed a set of genes whose expression is down-regulated in parallel with PAX3/FKHR, and was therefore analyzed in more detail.

Interestingly, at 24hrs after silencing only downregulated genes were identified which is in agreement with the observation that PAX3/FKHR mainly acts as transcriptional activator. Within the subset of potential PAX3/FKHR target genes, already known targets were present, like CB1 (Begum et al., 2005), MYCN (Khan *et al.*, 1998) and NCAM (Edelman and Jones, 1995), thus confirming our strategy. To constrict the list to those genes relevant in vivo, the set was compared to a PAX3(7)/FKHR-translocation specific gene signature identified directly from aRMS tumor biopsies. From this comparison, we identified a subset of 51 overlapping genes which are likely to represent relevant PAX3/FKHR targets important for the oncogenic properties of the fusion protein. The comparative expression profiling therefore revealed a large amount of novel biological information. In a very recent study, Davicioni et al. measured the expression profiles after expression of PAX3/FKHR in the related embryonal RMS cell line RD and compared this gene set to data from tumor biopsies (Davicioni *et al.*, 2006). Of the 61 potential target genes identified in their study, 9 are identical with genes identified in our study, namely ABAT, ADAM10, BMP5, IL4R, KIAA0555, MYCN, NELL1, NRCAM and POU4F1. In addition, another three genes (TFAP2B, CDH3 and CNR1) were excluded in their analysis only because transcriptional activation in RD cells was below the defined threshold level. This again underscores the validity of our approach.

Performing ontology studies, six main groups of target genes were identified, such as genes encoding for receptors, among them FGFR2 or IL4R, which are obvious candidates as therapeutic targets. Moreover, genes

like NCAM, ADRA2A, POU4F1 or BMP5 could elucidate pathways involved in cell development and differentiation in aRMS. Other characteristics of aRMS tumors may be the result of other targets of PAX3/FKHR, such as ADAM 10 and ADAM 19 which could play a role in enhanced metastatic potential of aRMS cells, a crucial property of the alveolar subtype.

Interestingly, 72hrs after siRNA treatment a set of genes upregulated in Rh4, but not in RD cells was identified, suggesting a specific repressing effect of PAX3/FKHR. This set included numerous genes related to muscle differentiation (see Table 2). There are different possible explanations for this observation: first, PAX3/FKHR could upregulate a transcriptional repressor, whereby repression would be an indirect effect, consistent with the time point (72hrs) at which expression changes were identified. Alternatively PAX3/FKHR is directly involved in repressing myogenic differentiation, which is consistent with the oncogenic role of this transcription factor. This differentiation-repressing activity of PAX3/FKHR is supported by recent studies, where PAX3 was shown to play a role in both initiation of the melanogenic cascade while preventing at the same time terminal differentiation in melanocyte stem cells (Lang *et al.*, 2005). The precise mechanism how PAX3/FKHR accomplishes this differentiation barrier in aRMS is not clear. However, in our study expression levels of well known factors in the myogenic differentiation pathway downstream of PAX3 such as myogenin or MyoD were to low to be detected on our microarrays.

Among the target genes identified which are interesting candidates to transduce the oncogenic effects of PAX3/FKHR was TFAP2B. It belongs to a transcription factor family consisting of four members, which are known to be coexpressed in early premigratory and migrating neural crest cells. Moreover, TFAP2B has been shown to play a role in apoptosis and survival of epithelial cells in collecting ducts and distal tubuli in mice embryonic tissue (Moser *et al.*, 2003; Moser *et al.*, 1997). Since PAX3/FKHR is also involved in regulation of cell survival, TFAP2B was confirmed on the molecular level as a direct target of PAX3/FKHR. The use of PAX3/FKHR mutants with impaired DNA-binding activity demonstrated paired domain dependency of TFAP2B expression, and promoter studies identified two paired domain binding sites in the TFAP2B promoter.

Further supporting the notion that TFAP2B is a physiologically relevant in vivo target gene comes from the recent observation that TFAP2B is a highly specific and sensitive marker for translocation-positive aRMS in immunohistochemical analysis (Wachtel *et al.*, 2006). This study directly confirms in vivo expression of the TFAP2B protein in a large number of tumor samples. Importantly, a similar behaviour was observed for CDH3 (p-cadherin), and also this gene was identified in our study. Therefore it very likely represents an additional in vivo target gene of PAX3/FKHR.

The physiological relevance of TFAP2B for aRMS cell growth and survival was directly examined next. Our data show that downregulation of TFAP2B in aRMS cells induced apoptosis as efficiently as downregulation of PAX3/FKHR. Interestingly, induction of apoptosis by silencing of PAX3/FKHR could be prevented by TFAP2B overexpression. These results suggest that TFAP2B is directly involved in transduction of a PAX3/FKHR regulated oncogenic characteristic namely anti-apoptotic properties. Identification and analysis of the downstream apoptotic mechanisms is currently ongoing and may identify additional therapeutic target genes. In addition, these rescue experiments directly demonstrate that our target gene signature, is not due to any off-target effects of siRNA treatment.

In conclusion, we identified a comprehensive signature of in vivo PAX3/FKHR target genes which are likely involved in mediating several oncogenic properties of the fusion protein such as migration, differentiation and survival. Indeed, TFAP2B mediates, at least in part, the survival function of PAX3/FKHR. Our approach of silencing fusion genes in its cellular context combined with in vivo expression data appears to be highly successful for identification of physiological targets to develop new therapeutics.

Material and methods

Cell lines and plasmids

Rh4 alveolar rhabdomyosarcoma (aRMS) cells were kindly provided by Peter Houghton (St. Jude Children's Research Hospital, Memphis, TN, USA). RD embryonal rhabdomyosarcoma (eRMS) and 293T human embryonic kidney cells were obtained from ATCC (LGC Promochem, Molsheim Cedex, France).

The PAX3/FKHR construct consists of 3.7 kb insert cloned into pcDNA3 vector, PAX3/FKHR derived mutants have a single point mutation G48S or N269A located in the paired and homeodomain, respectively.

For generation of Rh4 cells stably overexpressing murine TFAP2B, cells were transfected with the pcDNA3.1Neo plasmid containing a 1.8 kb TFAP2B insert. Mock-transfection with pcDNA3.1Neo was performed in parallel as control. Selection of stably transfected cells was preformed with 1 mg/ml G-418 Sulfate (Promega).

siRNA-mediated silencing

PAX3 and PAX3/FKHR knockdown was induced by RNA interference (RNAi) (Elbashir *et al*., 2001). A total of $2x10^5$ Rh4 or RD cells was plated and 24hrs later transfected with a combination of two chemically synthesized siRNAs (5'AAGAGAGAACCCGGGCAUG-dTdT, and 5'CAUGGAUUUUCCAGCUAUA-dTdT) both targeting the PAX3 part of the fusion gene (Qiagen, Hombrechtikon, Switzerland). For downregulation of TFAP2B, Rh4 cells were transfected with siRNA with the sequence 5'ACUUCGAAGUACAAAGUAA-dTdT (Qiagen, Catalog No. S100049259). As positive control siRNA targeting GAPDH (Catalog No. 4605, Ambion, Huntingdon, United Kingdom) was used, as negative control scrambled siRNA (scRNA) with the sequence 5'UUCUUCGAACGUGUCACGU-dTdT (Qiagen, Catalog No. 1022076) with no known homology to mammalian genes. Transfection was done according to the manufacturer's instructions using 7µl of GeneEraser (Stratagene, La Jolla, CA) and 20nM siRNA (final concentration).

Quantitative RT-PCR

Total RNA (1µg) was reverse-transcribed with Oligo$(dT)_{15}$ Primer using the Omniscript Reverse Transcription Kit (Qiagen). Quantitative RT-PCR (qRT-PCR) detection of PAX3, TFAP2B and GAPDH was carried out with the commercially available assays-on-demand Hs00240950_m1, Hs00231468_m1 and Hs99999905_m1 (Applied Biosystems, Rotkreuz, Switzerland), respectively. QRT-PCR detection of PAX3/FKHR was performed using PAX3 FOR (5'GCACTGTACACCAAAGCACG3') and FKHR REV

(5'AACTGTGATCCAGGGCTGTC3') primers applying the fluorescent SYBR green method (Applied Biosystems) on an Applied Biosystems 7900HT.

Western Blot

10µg of total nuclear protein was used for western blotting using NuPAGE electrophoresis system (Invitrogen, Basel, Switzerland). For PAX3 detection a goat-anti-PAX3 antibody (Santa Cruz Biotechnology, Heidelberg, Germany), for PAX3/FKHR detection a rabbit-anti-FKHR antibody (Cell Signaling Technology, Allschwil, Switzerland) and for detection of TFAP2B protein, a mouse-anti-TFAP2B antibody (Abcam, Cambridge, UK) were used.

Gene expression analysis

Global changes in gene expression were measured using Affymetrix HG-133A GeneChip arrays (Affymetrix Inc., Santa Clara, CA). cRNA target synthesis and experimental procedures for GeneChip hybridization and scanning were carried out according to the "GeneChip eukaryotic small sample target labeling technical note" (Affymetrix, Santa Clara, CA). Expression data of siRNA-, scRNA (control) – and non-treated cells was analyzed using dChip2004(Li & Wong, 2001) and GeneSpring7.0 with default normalization and a cross-gene-error model, resulting in 16221 genes. Representative data from two biological replicates is shown.

Cell Proliferation assays

Cell proliferation was measured using the MTT assay (Roche, Rotkreuz, Switzerland). Prior to MTT measurement, a standard curve for each cell line was generated using 500 to 1x 10^5 cells per well. A total of $1x10^4$ Rh4 or RD cells were plated per 96-well and transfected 24h later. The amount of converted MTT reagent was measured at different time points up to 72h later by a multi-detection microplate reader (Bio-Tek Instruments, Inc., Littau, Switzerland).

Apoptosis assays

One thousand cells from each experimental condition were assayed for caspase-3 activation using the Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's instructions. Caspase activity was measured at an excitation wavelength of 485nm and an emission wavelength of 516nm. For the calculation of standard deviations, first the quotient of treated versus untreated cells was determined. The standard deviation of the quotient was then calculated as follows: for two numbers, A and B, with standard deviations, a and b,

$(A \pm a) / (B \pm b) = (C \pm c)$ and $c = A/B * \text{sqrt}((a/A)^2 + (b/B)^2)$ where * indicates multiplication.

For FACS analysis, Rh4 cells from one confluent 35mm dish were stained with 200µl of Propidium Iodide (PI) followed by cytometry analysis on a Cytomics FC500 Instrument (Beckman Coulter, Nyon, Switzerland). The flow cytometry data was then analysed by the FlowJo software.

Cloning of the TFAP2B promoter and generation of deletion constructs

TFAP2B promoter region from positions -3200 to +1 was amplified by PCR (primer FOR 5'AAAGTACGAGTGTTAACTATCTGG3'; REV 5'GCAGCCTGGTCTCTAGGAGG3') from 293T cell genomic DNA and cloned into the pGL3basic luciferase vector (Promega, Wallisellen, Switzerland). Deletion constructs were prepared using the Erase-a-base Kit (Promega), allowing progressive unidirectional deletions of approximately 200 bp at the 5' end of the insert.

Luciferase assay

A total of $1x10^5$ 293T cells were plated per 35mm plate and cotransfected 24hrs later with 2 µg of TFAP2B-promoter in pGL3basic plasmid plus 1 µg of either PAX3/FKHR or PAX3/FKHR derived mutants or pcDNA empty vector plus 100ng of pFIV-CMV-LacZ control plasmid (System Biosciences, Heidelberg, Germany) using the Ca_2PO_4 transfection method. 24h posttransfection, cells were lysed in reporter lysis buffer and assayed for luciferase as well as β-galactosidase activity using the corresponding assay systems (Promega). Luciferase activity values were normalized to the ß-galactosidase activity and expressed as relative luciferase units.

Chromatin Immunoprecipitation (ChIP) assay

ChIP was performed using a commercially avaible ChIP-IT enzymatic kit (Active Motif, Rixensart, Belgium) according to manufacturer's instructions. 293T cells were cotransfected with two different deletion constructs, TFAP2b_1 or TFAP2b_2 containing 1592bp and 806bp of the TFAP2B upstream promoter region, respectively and a PAX3 construct encoding for His-tagged PAX3 protein. DNA-bound protein was immunoprecipitated using an anti-His (Quiagen) antibody or mouse IgG (Active Motif) as negative control. For quantification of coprecipitated DNA, amplification of a 470bp region of the TFAP2beta promoter with primer FOR 5'GCGCAGAGATCCTCTTCTGG3' and REV 5'AGCAACGTACGCACACGTTC3' was measured by Sybr Green qRT-PCR. Signals of the anti-His precipitates were normalizes to the signals of the IgG precipitates.

Electrophoretic Mobility Schift Assays (EMSA)

EMSA were performed using the Chemiluminescent Nucleic Acid Detection Module (Pierce, Rockford, USA) according to manufacturer's instructions. Each protein-DNA binding reaction was carried out using 8µl of nuclear extracts from PAX3-His or PAX3/FKHR transiently transfected 293T cells and 20fmol of biotin labeled double stranded oligonucleotides corresponding to two possible PAX3 binding sites. Deletion site2 specific sequences Del2 (5'AGATCCTCTTCTGGGCGTCTGTTCCGGCTATGAGAAGCTCTCCGCA3') and as control Del2Contr (5'AGATCCTCTTCTGGGCGTCTAAAAAAGCTATGAGAAGCTCTCCGCA3') as well as deletion site3 specific sequences Del3 (5'GGGGATGGGAAAGGGGGAACAGGGGAACAGATGAGTATTCATTTC3') and the control Del3Contr (5'GGGGATGGGAAAGGGAAAAAAAGAAAAAAGATGAGTATTCATTTC3') were synthesized as 5' Biotin-labeled complementary oligonucleotide pairs (Microsynth, Balgach, Switzerland).

Acknowledgments

We thank F.G. Barr (University of Pennsylvania Philadelphia) and Prof. R. Fässler (Max-Planck-Institute, Münich, Germany) for providing cDNA constructs, A. Patrignani (FGCZ) for excellent technical assistance with Affymetix experiments and Dr. M. Dettling for performing principal component analysis. This work was supported by Swiss National Science Foundation, grant 3100-067841 and 3100-109837 and the Schweizerische Forschugsstiftung Kind und Krebs.

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Figure legends

Figure 1 Physiological effects of PAX3 and PAX3/FKHR silencing in RMS cells.

(A) mRNA levels of PAX3 and PAX3/FKHR as measured by qRT-PCR analysis after RNAi mediated downregulation in eRMS (RD) and aRMS (Rh4) cells. The ratio of siRNA to scRNA (control) treatment is shown in %. (B) Protein levels as detected by Western blot in 10 µg of nuclear extract per lane. PAX3/FKHR levels were detected in Rh4 cells, PAX3 levels were detected in RD cells. Cells were treated as indicated with PAX3 specific siRNA (si) or control scRNA (sc). (C, D) Proliferation of RD and Rh4 cells as measured by MTT assay after 72h of treatment with either PAX3 specific siRNA, scRNA, siRNA targeting GAPDH, or transfection reagent alone (GeneEraser). The means ±standard deviations (error bars) from three independent triplica experiments are shown. (E, F) Induction of apoptosis in RD and Rh4 cells after the indicated time periods of treatment with either PAX3 specific siRNA, scRNA, siRNA targeting GAPDH or GeneEraser (transfection reagent) as measured by caspase-3/7 assay. Fold change values of caspase activity of treated vs. non-treated cells from two independent triplica experiments are shown.

Figure 2 Changes in gene expression after PAX3/FKHR silencing and identification of PAX3/FKHR target genes in aRMS cells.

(A) Diagram depicting normalized expression values (log10 scale) of 16221 filtered genes as measured in Rh4 cells after 0, 24, 48 and 72hrs of treatment with PAX3-specific siRNA. Each line represents the expression of one gene. (B) Diagram depicting the overlap between a PAX3(7)/FKHR-specific gene signature derived from aRMS biopsies and genes downregulated by PAX3-specific siRNA treatment in Rh4 cells: 51 genes are present in both signatures.

(C) Temporal changes of expression levels of the 51 genes identified in B in Rh4 cells after 0, 24, 48 and 72hrs of treatment with PAX3-specific siRNA or scRNA. (D) Graphic representation depicting the ontology of the 51 potential PAX3/FKHR target genes identified in B. A complete list of the functions as well as downregulation levels of the 51 genes is shown in Tab. 1.

Figure 3 Upregulated genes after silencing of PAX3 and PAX3/FKHR in RMS cells.

(A) Diagram depicting expression values (fold change scale) of 260 genes upregulated more than 2-fold in Rh4 cells after 72h of PAX3-specific siRNA treatment. (B) Expression levels of the same set of genes as in A in RD

cells. Expression values were calculated in relation to scRNA treatment, levels of upregulation are listed in supplementary Tab. 2.

(C) Diagram depicting the expression values (fold change scale) of 52 genes (represented by 67 probe sets) involved in muscle development, which are upregulated in Rh4 cells after silencing of PAX3/FKHR for 72hrs. Levels of upregulation are listed in Tab.2 (D) Diagram depicting the expression values of the same 52 genes as in C in RD cells after 72hrs of PAX3 silencing. E, F: Verification of the expression changes of two genes, TNNC2 and MYL1, by qRT-PCR analysis. Fold change values in siRNA - compared to scRNA-treated cells is shown.

Figure 4 Targeting of the TFAP2b promoter by PAX3/FKHR.

(A) mRNA levels of TFAP2b and CB1 were measured by qRT-PCR in Rh4 cells at indicated time points after RNAi mediated PAX3/FKHR downregulation. The ratio of siRNA to scRNA treatment is shown in %. (B) mRNA levels of TFAP2b and CB1 in 293T cells, either untransfected or transfected with the indicated PAX3/FKHR construct, as measured by qRT-PCR. PAX3hd and PAX3pd are homeodomain- and paired domain-specific mutants of PAX3/FKHR. (C) Deletion analysis of the TFAP2B promoter. A luciferase reporter construct containing the 3200 bp upstream of the TFAP2B transcriptional start site (TFAP2b_full) or deletion constructs thereof, containing remaining parts of 1592bp (TFAP2b_1) or 806bp (TFAP2b_2), respectively, are shown schematically in the upper panel. Transactivation of PAX3/FKHR on the different constructs as measured by luciferase assays is shown in the lower panel. The means ±standard deviations (error bars) from two independent duplicate experiments are shown ([RLU], relative light units). (D) Transactivation of the indicated PAX3/FKHR constructs on deletion construct TFAP2b_1 as measured by luciferase assay. (E) Sequences of three potential PAX3/FKHR binding sites within the TFAP2B promoter region. Commonly described motifs for paired domain binding are indicated by bold cursive type. Sequences for the design of oligonucleotides used for EMSA are underlined. (F) Binding of PAX3 to the promoter regions of the TFAP2b_1 and TFAP2b_2 deletion constructs as detected by chromatin immunoprecipitation. His-tagged PAX3 protein/DNA complexes were immunoprecipitated with αHis antibody or unspecific mouse αIgG. QRT-PCR quantification of precipitated DNA of each experiment (n=3) was performed in triplicates and values from the anti-His immunoprecipitation were normalized with the values from the control immunoprecipitation with IgG. (G) Schematic representation of single deletions of possible binding sites of PAX3/FKHR in TFAP2b_1 as introduced by site-directed mutagenesis is shown in the upper panel. Luciferase activity of the indicated deletion constructs as measured after cotransfection with PAX3/FKHR in 293T cells. Each bar represents means ±standard deviations from three

independent duplicate experiments. (H) EMSA used for measurement of DNA binding properties of PAX3 against the deletion site 2 in the promoter region of TFAP2B. The assay was performed with 8 µl of nuclear protein extracted after transfection of PAX3-His in 293T cells. As control, mock-transfected 293T cells and mismatch oligonucleotides were used.

Figure 5 Physiological effects of TFAP2B silencing in aRMS cells.

(A) mRNA level of TFAP2B after RNAi mediated downregulation in Rh4 cells as measured by qRT-PCR analysis. The ratio of siRNA to scRNA (control) treatment is shown in %. (B) Protein levels of TFAP2B as determined by Western Blot with 10 µg of nuclear protein from TFAP2B specific - (si) and control siRNA (sc) treated cells extracted 24h and 48h after transfection. In the positive control lane, mTFAP2B transiently overexpressed in 293T cells is shown as size control. FKHR protein levels were used as loading control. (C) Cell proliferation as measured by MTT assay after different time periods of treatment with TFAP2B specific siRNA, scRNA and transfection reagent alone (GeneEraser). The means ±standard deviations (error bars) from three independent triplicates experiments are shown. (D) Apoptosis rate in Rh4 cells transfected with the indicated siRNA as detected by a caspase-3/7 assay at the indicated time points. Mean values of one representative experiment performed in triplicates are shown. (E) Histogram plots of FACS results of cells stained with PI 24h after treatment with indicated the siRNA. Percentages of PI-positive cells are depicted above the horizontal bars.

Figure 6 Physiological link between PAX3/FKHR and TFAP2B.

(A) Cell proliferation of mock-transfected Rh4 cells and cells stably overexpressing mTFAP2B as measured by MTT assay at indicated time points after PAX3/FKHR silencing. Treatment with siRNA specific for PAX3/FKHR was compared to scRNA, siRNA targeting GAPDH and GeneEraser alone. (B) Comparison of apoptosis rates in parental Rh4 cells, Rh4 cells stably overexpressing TFAP2B and mock-transfected Rh4 cells as measured by FACS of PI-stained cells 24h after downregulation of PAX3/FKHR.

Figure 1

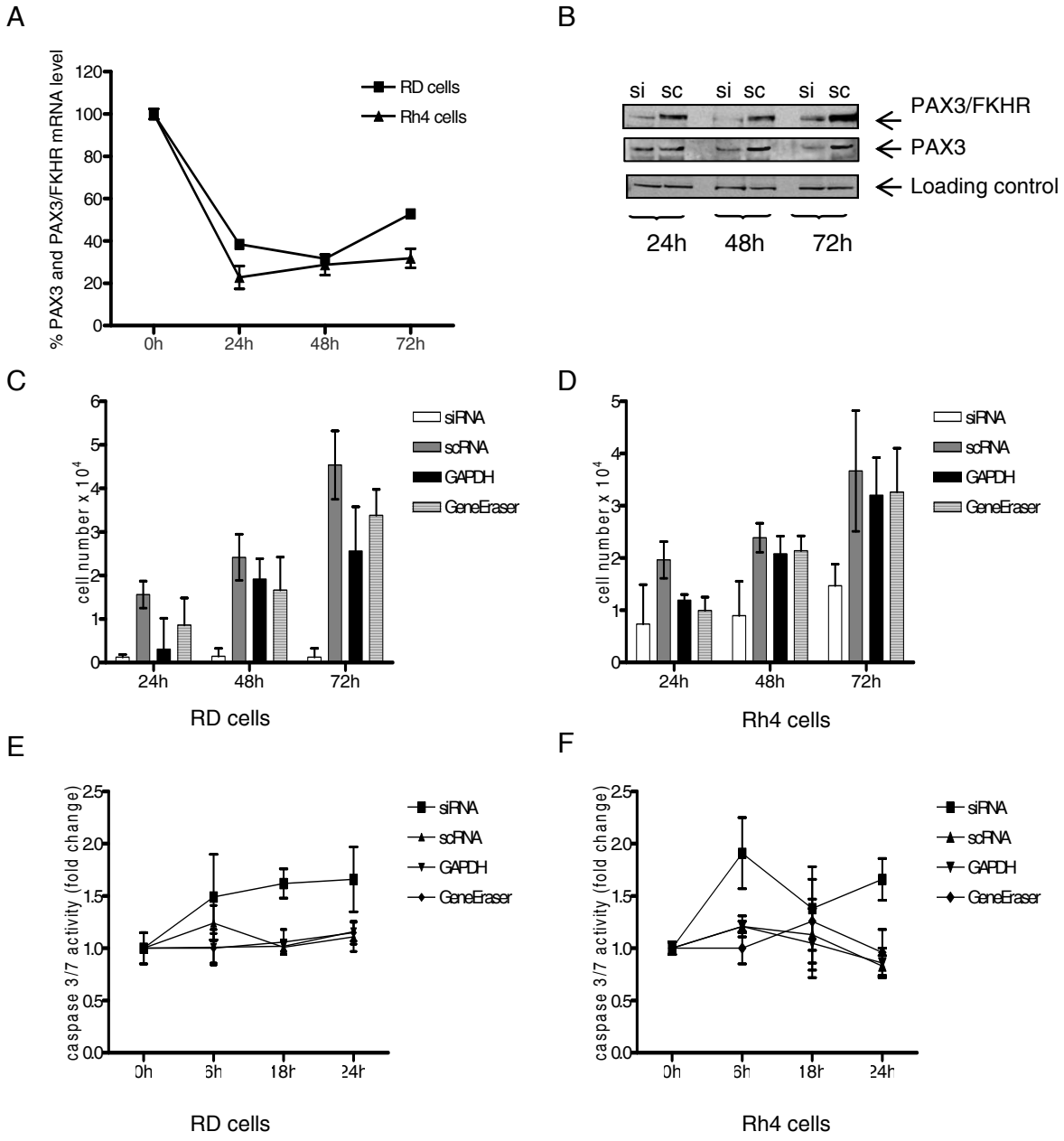
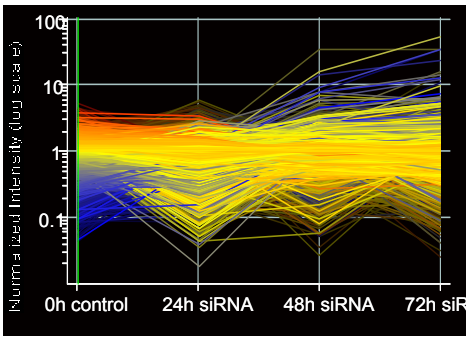

A
% PAX3 and PAX3/FKHR mRNA level
RD cells
Rh4 cells
0h
24h
48h
72h
B
si sc si sc si sc
PAX3/FKHR
PAX3
Loading control
24h
48h
72h
C
cell number x 10^4
siRNA
scRNA
GAPDH
GeneEraser
RD cells
D
Rh4 cells
E
caspase 3/7 activity (fold change)
RD cells
F
Rh4 cells

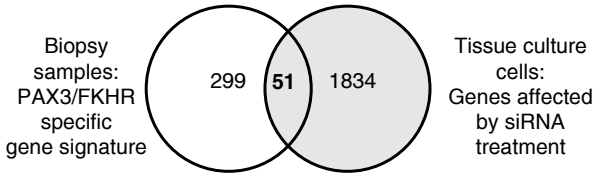
Figure 2

A

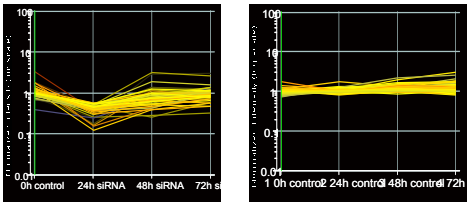


Rh4 cells

B



C



siRNA scRNA

D

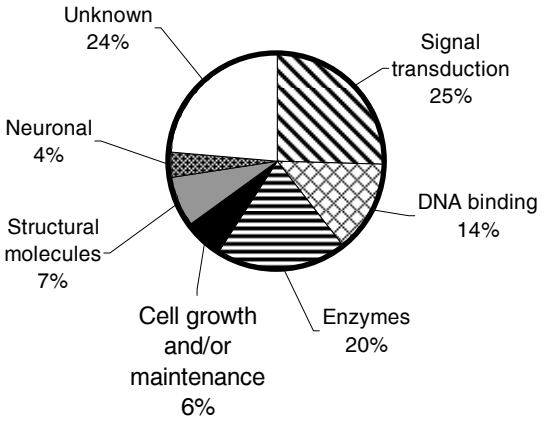
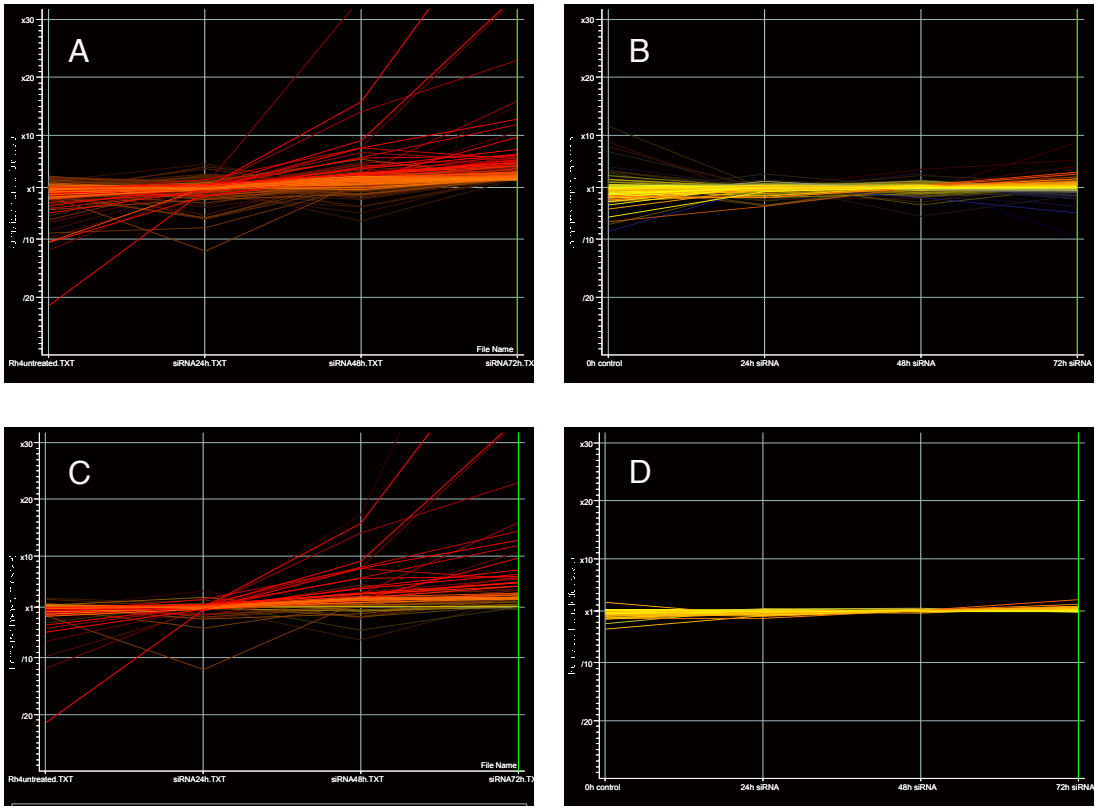
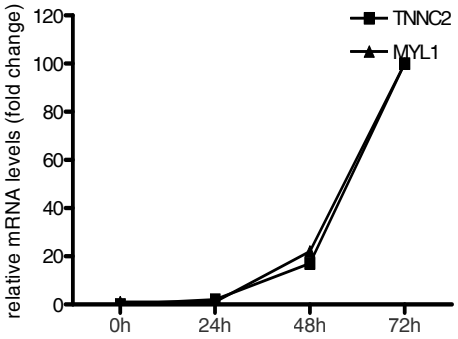


Figure 3


A
B
C
D
x30
x20
x10
x1
/10
/20
Rh4untreated.TXT
siRNA24h.TXT
siRNA48h.TXT
siRNA72h.TXT
File Name
0h control
24h siRNA
48h siRNA
72h siRNA
Rh4 cells
RD cells

E


relative mRNA levels (fold change)
120
100
80
60
40
20
0
TNNC2
MYL1
0h
24h
48h
72h
Rh4 cells

F

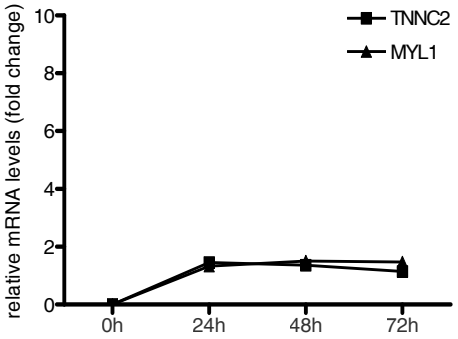

relative mRNA levels (fold change)
10
8
6
4
2
0
TNNC2
MYL1
0h
24h
48h
72h
RD cells

Figure 4

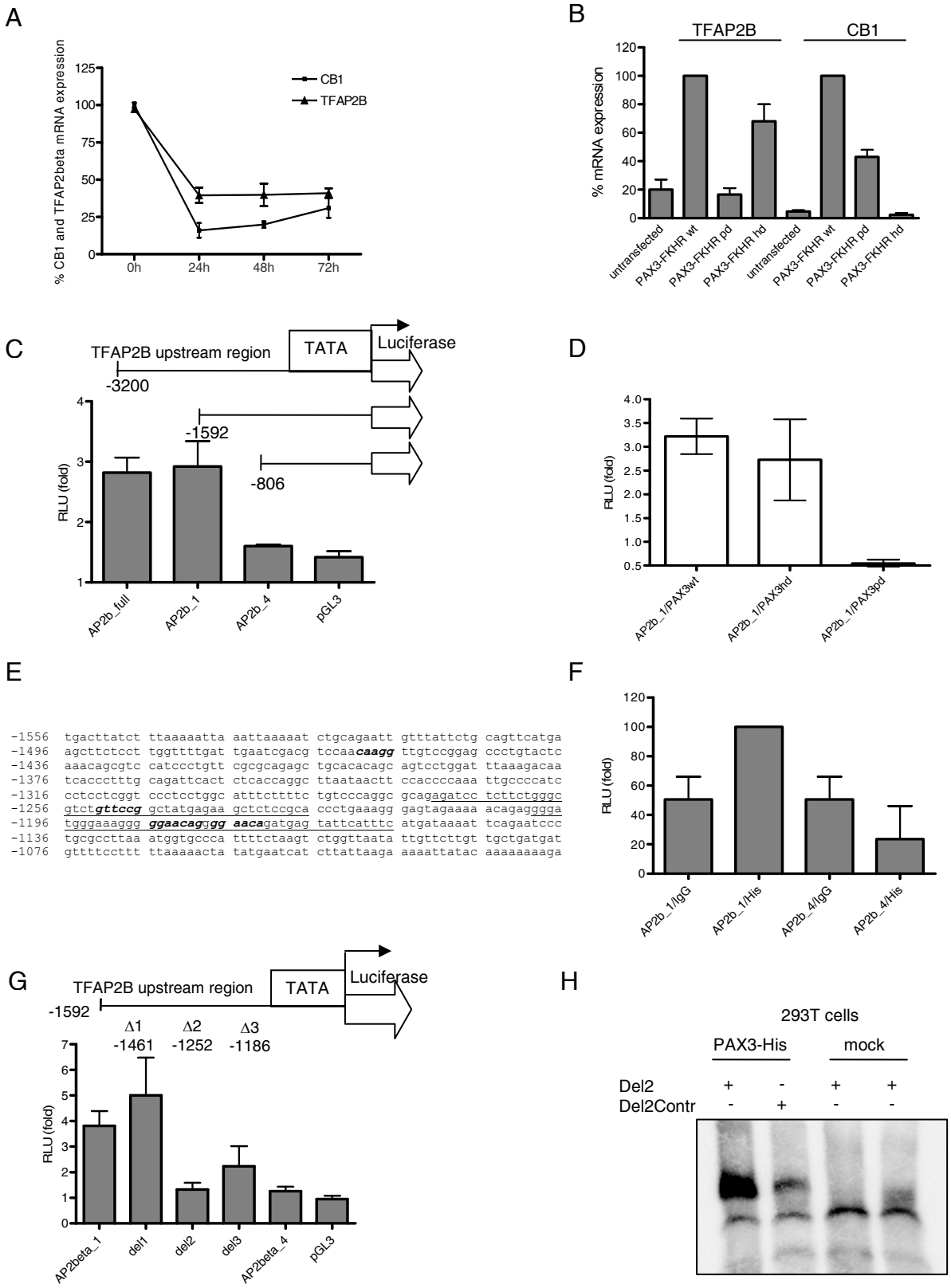

A
% CB1 and TFAP2beta mRNA expression
CB1
TFAP2B
0h
24h
48h
72h
B
TFAP2B
CB1
% mRNA expression
untransfected
PAX3-FKHR wt
PAX3-FKHR pd
PAX3-FKHR hd
C
TFAP2B upstream region
TATA
Luciferase
-3200
-1592
-806
RLU (fold)
AP2b_full
AP2b_1
AP2b_4
pGL3
D
RLU (fold)
AP2b_1/PAX3wt
AP2b_1/PAX3hd
AP2b_1/PAX3pd
E
-1556 tgacttatct ttaaaaatta aattaaaaat ctgcagaatt gtttattctg cagttcatga
-1496 agcttctcct tggttttgat tgaatcgacg tccaacaagg ttgtccggag ccctgtactc
-1436 aaacagcgtc catccctgtt cgcgcagagc tgcacacagc agtcctggat ttaaagacaa
-1376 tcacccttg cagattcact ctcaccaggc ttaataactt ccaccccaaa ttgccccatc
-1316 cctcctcggt ccctcctggc atttcttttc tgtcccaggc gcagagatcc tcttctgggc
-1256 gtctgttccg gctatgagaa gctctccgca ccctgaaagg gagtagaaaa acagagggga
-1196 tgggaaaggg ggaacaggg aacagatgag tattcatttc atgataaaat tcagaatccc
-1136 tgcgccttaa atggtgccca ttttctaagt ctggttaata ttgttcttgt tgctgatgat
-1076 gttttccttt ttaaaaacta tatgaatcat cttattaaga aaaattatac aaaaaaaaga
F
RLU (fold)
AP2b_1/IgG
AP2b_1/His
AP2b_4/IgG
AP2b_4/His
G
TFAP2B upstream region
TATA
Luciferase
-1592
Δ1
-1461
Δ2
-1252
Δ3
-1186
RLU (fold)
AP2beta_1
del1
del2
del3
AP2beta_4
pGL3
H
293T cells
PAX3-His
mock
Del2
Del2Contr

Figure 5

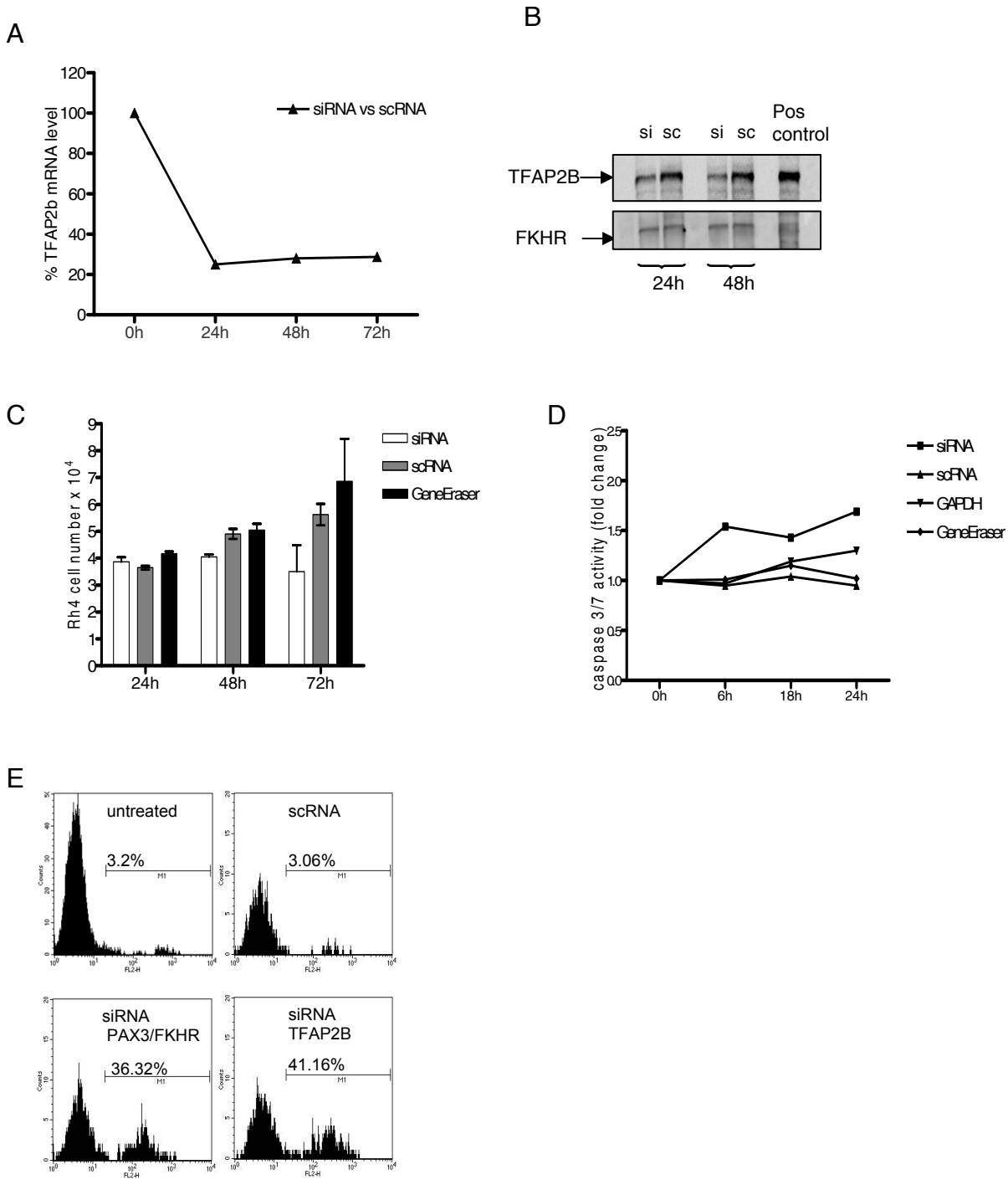
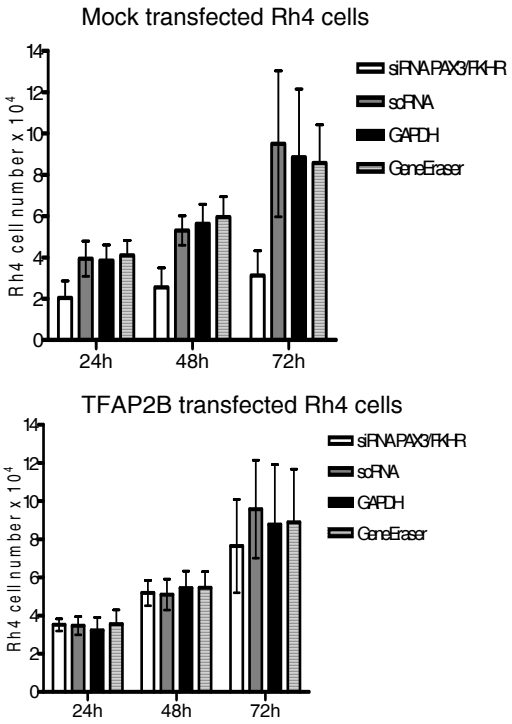

A
% TFAP2b mRNA level
siRNA vs scRNA
0h
24h
48h
72h
B
si sc si sc Pos control
TFAP2B
FKHR
24h
48h
C
Rh4 cell number x 10^4
siRNA
scRNA
GeneEraser
24h
48h
72h
D
caspase 3/7 activity (fold change)
siRNA
scRNA
GAPDH
GeneEraser
0h
6h
18h
24h
E
untreated
3.2%
scRNA
3.06%
siRNA PAX3/FKHR
36.32%
siRNA TFAP2B
41.16%

Figure 6

A



B

Cell line	Treatment	Apoptosis rate	
		%PI positive cells	Fold change siRNA vs control
Rh4	PAX3/FKHR silencing	46%	**5.1**
Rh4	control	9%	
Rh4 mock	PAX3/FKHR silencing	35%	**3.5**
Rh4 mock	control	10%	
Rh4 mTFAP2B	PAX3/FKHR silencing	21%	**1.6**
Rh4 mTFAP2B	control	13%	

I. Table1 Ontology and downregulation levels of 51 potential PAX3/FKHR target genes

Gene Name	Common	Description	Downregulation 24h (fold)	Function
Signal transduction				
207232_s_at	DZIP3	zinc finger DAZ interacting protein 3	6.1	ligase activity, protein ubiquitination, RNA binding, zinc ion binding
220197_at	ATP6V0A4	ATPase, H+ transporting, lysosomal V0 subunit a isoform 4	5.7	ion transport, proton transport , regulation of pH
204882_at	ARHGAP25	Rho GTPase activating protein 25	4.2	GTPase activator activity
203638_s_at	FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	3.2	cell growth, protein amino acid phosphorylation
206723_s_at	EDG4	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 4	2.9	G-protein coupled receptor protein signaling pathway, lysosphingolipid and lysophosphatidic acid receptor activity, lipid binding
212915_at	SEMACAP3	Likely ortholog of mouse semaF cytoplasmic domain associated protein 3	2.7	metal ion binding, protein ubiquitination, ubiquitin-protein ligase activity
203233_at	IL4R	interleukin 4 receptor	2.6	immune response,receptor signaling protein activity
209869_at	ADRA2A	adrenergic, alpha-2A-, receptor	2.5	activation of MAPK activity, Ras protein signal transduction, positive regulation of cell proliferation
213436_at	CNR1; CB1; CB-R; CB1A; CANN6; CB1K5	cannabinoid receptor 1 (brain)	2.3	cannabinoid receptor activity, G-protein signaling, coupled to cyclic nucleotide second messenger
205068_s_at	GRAF	GTPase regulator associated with focal adhesion kinase pp125(FAK)	1.9	nervous system development, Rho GTPase activator activity
221578_at	RASSF4	Ras association (RalGDS/AF-6) domain family 4	1.8	oxidoreductase activity
200972_at	TM4SF8	transmembrane 4 superfamily member 8	1.7	cell motility, cell proliferation
218373_at	FTS	fused toes homolog (mouse)	1.6	apoptosis, ubiquitin-like activating enzyme activity
DNA binding				
202320_at	GTF3C1	general transcription factor IIIC, polypeptide 1, alpha 220kDa	9.6	DNA binding, RNA polymerase III transcription factor activity, rRNA transcription, tRNA transcription
205935_at	FOXF1	forkhead box F1	2.8	regulation of transcription, DNA-dependent; regulation of transcription from Pol II promoter
209757_s_at	MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	2.8	transcription factor activity, protein binding, regulation of transcription, DNA-dependent
206940_s_at	POU4F1	POU domain, class 4, transcription factor 1	2.7	development, transcription factor activity, regulation of transcription from Pol II promoter
218445_at	H2AFY2	H2A histone family, member Y2	2.6	dosage compensation
214451_at	TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta)	2.6	nervous system development, transcription factor activity, transcription coactivator activity
211341_at	POU4F1	POU domain, class 4, transcription factor 1	2.4	development, transcription factor activity, regulation of transcription from Pol II promoter
Enzymes				
212989_at	MOB	mob protein	3.4	kinase activity, lipid metabolism, transferase activity

214961_at	KIAA0774	KIAA0774	3.1	catalytic activity
221605_s_at	PIPOX	pipecolic acid oxidase	3.0	electron transport, oxidoreductase activity, tetrahydrofolate metabolism
206447_at	ELA1	elastase 1, pancreatic	3.0	trypsin activity, pancreatic elastase activity, hydrolase activity
209460_at	ABAT	4-aminobutyrate aminotransferase	2.9	transferase activity, neurotransmitter catabolism
209765_at	ADAM19	a disintegrin and metalloproteinase domain 19 (meltrin beta)	2.5	hydrolase activity; metalloendopeptidase activity, proteolysis
212006_at	UBXD2	UBX domain containing 2	2.3	regulation of transcription, DNA-dependent , transcription factor activity
204074_s_at	KIAA0562	glycine-, glutamate-, thienylcyclohexylpiperidine-binding protein	2.2	binding, hydrolase activity
205811_at	POLG2	polymerase (DNA directed), gamma 2, accessory subunit	1.9	DNA repair, DNA-dependent DNA replication, protein biosynthesis, metallopeptidase activity
202603_at	ADAM10	a disintegrin and metalloproteinase domain 10	1.9	hydrolase activity, protein amino acid phosphorylation, metalloendopeptidase activity
Cell growth and/or maintenance				
205430_at	BMP5	Human DNA sequence from clone RP1-181C24 on chromosome 6p11.1-12.2 Contains the 3' end of the BM	6.2	cell differentiation, cytokine activity, growth, growth factor activity, skeletal development
209814_at	ZNF330	zinc finger protein 330	2.9	metal ion binding, protein binding , zinc ion binding
203651_at	ZFYVE16	zinc finger, FYVE domain containing 16	1.9	endosome transport , metal ion binding, protein targeting to lysosome, regulation of endocytosis, vesicle organization and biogenesis
Structural molecules				
221854_at	PKP1	plakophilin 1 (ectodermal dysplasia/skin fragility syndrome)	8.0	cell-cell signaling, cell adhesion
203256_at	CDH3	cadherin 3, type 1, P-cadherin (placental)	6.0	cell adhesion, calcium ion binding,
203072_at	MYO1E	myosin IE	2.1	actin binding, ATP binding, ATPase activity, actin filament-based movement
211059_s_at	GOLGA2	golgi autoantigen, golgin subfamily a, 2	1.7	protein binding
Neuronal				
206089_at	NELL1	NEL-like 1 (chicken)	4.7	nervous system development, cell adhesion
204105_s_at	NRCAM	neuronal cell adhesion molecule	3.5	ankyrin binding, cell-cell adhesion, neuron migration, positive regulation of neuron differentiation, protein binding,synaptogenesis
Unknown				
210102_at	LOH11CR2A	loss of heterozygosity, 11, chromosomal region 2, gene A	2.7	unknown
221185_s_at	DKFZp434B2 27	synonyms: FLJ11667, FLJ23571; Homo sapiens hypothetical protein DKFZp434B227 (DKFZp434B227), mRNA.	2.6	unknown
205888_s_at	KIAA0555	KIAA0555 gene product	2.5	unknown
213179_at		602381329F1 NIH_MGC_93 Homo sapiens cDNA clone IMAGE:4499023 5', mRNA sequence.	2.4	unknown
209693_at	ASTN2	astrotactin 2	2.4	unknown
219438_at	FLJ12650	hypothetical protein FLJ12650	2.3	unknown
220610_s_at	LRRFIP2	leucine rich repeat (in FLII) interacting protein 2	2.3	unknown

207759_s_at	FLJ41105	hypothetical gene supported by AK123100	2.2	unknown
49111_at		MRNA; cDNA DKFZp762M127 (from clone DKFZp762M127)	2.2	unknown
43511_s_at		MRNA; cDNA DKFZp762M127 (from clone DKFZp762M127)	1.9	unknown
212736_at	BC008967	hypothetical gene BC008967	1.8	unknown
219225_at	PGBD5	piggyBac transposable element derived 5	1.6	unknown

II Table 2 Upregulation levels of 52 muscle development related genes represented by 67 probe sets

Gene Name	Common	Description	Rh4 fold change upregulation si vs sc	RD fold change upregulation si vs sc
209904_at	TNNC1	troponin C, slow	58.2	0.8
205163_at	HUMMLC2B	myosin light chain 2	37.1	0.9
205388_at	TNNC2	troponin C2, fast	34.4	1.0
206304_at	MYBPH	myosin binding protein H	22.4	1.0
205940_at	MYH3	myosin, heavy polypeptide 3, skeletal muscle, embryonic	21.6	0.6
206393_at	TNNI2	troponin I, skeletal, fast	18.4	0.8
221994_at	PDLIM5	LIM protein (similar to rat protein kinase C-binding enigma)	15.1	0.9
214122_at	PDLIM7	PDZ and LIM domain 7 (enigma)	14.2	1.2
210329_s_at	SGCD	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)	12.6	1.3
209283_at	CRYAB	crystallin, alpha B	9.6	1.0
205951_at	MYH1	myosin, heavy polypeptide 1, skeletal muscle, adult	9.5	5.1
219772_s_at	SMPX	small muscle protein, X-linked	8.6	1.1
205730_s_at	ABLIM3	Homo sapiens actin binding LIM protein family, member 3 (ABLIM3), mRNA.	8.3	0.9
206394_at	MYBPC2	myosin binding protein C, fast type	7.7	3.9
34471_at	MYH8	myosin, heavy polypeptide 8, skeletal muscle, perinatal	7.4	1.5
213023_at	UTRN	utrophin (homologous to dystrophin)	7.2	1.3
205610_at	MYOM1	myomesin 1 (skelemin) 185kDa	6.3	1.0
206116_s_at	TPM1	tropomyosin 1 (alpha)	6.2	0.7
209888_s_at	MYL1	myosin, light polypeptide 1, alkali; skeletal, fast	6.1	1.0
200974_at	ACTA2	actin, alpha 2, smooth muscle, aorta	5.9	0.8
218736_s_at	PALMD	palmdelphin	5.9	1.1
201438_at	COL6A3	collagen, type VI, alpha 3	5.8	1.2
206538_at	MRAS	muscle RAS oncogene homolog	5.6	3.5
210298_x_at	FHL1	four and a half LIM domains 1	5.3	1.0
210395_x_at	MYL4	myosin, light polypeptide 4, alkali; atrial, embryonic	5.3	0.6
216054_x_at	MYL4; GT1; ALC1; AMLC; PRO1957	Human MLC1emb gene for embryonic myosin alkaline light chain, promoter and exon 1.	5.2	0.5
205693_at	TNNT3	troponin T3, skeletal, fast	5.1	0.7
210088_x_at	MYL4	myosin, light polypeptide 4, alkali; atrial, embryonic	5.1	0.5
210330_at	SGCD	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)	5.0	2.1
206717_at	MYH8	myosin, heavy polypeptide 8, skeletal muscle, perinatal	5.0	1.4
217585_at	NEBL	nebulette	4.8	1.0
210202_s_at	BIN1	bridging integrator 1	4.6	0.8
205547_s_at	TAGLN	transgelin	4.5	0.8
203037_s_at	MTSS1	metastasis suppressor 1	4.2	1.0
207317_s_at	CASQ2	calsequestrin 2 (cardiac muscle)	4.1	1.1
213371_at	LDB3	LIM domain binding 3	4.1	0.7
203821_at	DTR	diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)	4.0	0.5
214087_s_at	MYBPC1	myosin binding protein C, slow type	4.0	1.0
203243_s_at	PDLIM5	LIM protein (similar to rat protein kinase C-binding enigma)	4.0	1.0
205177_at	TNNI1	troponin I, skeletal, slow	3.9	0.7
207302_at	SGCG	sarcoglycan, gamma (35kDa dystrophin-associated glycoprotein)	3.9	1.1
204173_at	MLC1SA	myosin light chain 1 slow a	3.8	0.9
206117_at	TPM1	tropomyosin 1 (alpha)	3.5	0.7
201976_s_at	MYO10	myosin X	3.4	1.0
202931_x_at	BIN1	bridging integrator 1	3.3	0.9
214492_at	SGCD	sarcoglycan, delta (35kDa dystrophin-	3.1	1.3

		associated glycoprotein)		
219829_at	ITGB1BP2	integrin beta 1 binding protein (melusin) 2	3.1	0.3
217274_x_at	MYL4; GT1; ALC1; AMLC; PRO1957	skeletal embryonic form; H.sapiens skeletal embryonic myosin light chain 1 (MLC1) mRNA	2.8	0.7
215279_at	SVIL	supervillin	2.7	1.1
213717_at	LDB3	LIM domain binding 3	2.6	1.0
204533_at	CXCL10	chemokine (C-X-C motif) ligand 10	2.5	1.2
213022_s_at	UTRN	utrophin (homologous to dystrophin)	2.5	1.4
214643_x_at	BIN1	bridging integrator 1	2.5	1.1
201079_at	SYNGR2	synaptogyrin 2	2.5	0.7
222022_at	DTX3	deltex 3 homolog (Drosophila)	2.5	1.2
215222_x_at		microtubule-actin crosslinking factor 1	2.4	0.8
210201_x_at	BIN1	bridging integrator 1	2.3	0.9
207968_s_at	MEF2C	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	2.3	1.0
209926_at	MEF2B	MADS box transcription enhancer factor 2, polypeptide B (myocyte enhancer factor 2B)	2.2	1.1
209200_at	MEF2C	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	2.2	1.0
219107_at	BCAN	brevican	2.2	1.1
216887_s_at	LDB3	LIM domain binding 3	2.2	1.0
210360_s_at	MTSS1	metastasis suppressor 1	2.1	0.7
206813_at	CTF1	cardiotrophin 1	2.1	0.8
202222_s_at	DES	desmin	2.1	0.7
203004_s_at	MEF2D	MADS box transcription enhancer factor 2, polypeptide D (myocyte enhancer factor 2D)	2.1	0.8
201792_at	AEBP1	AE binding protein 1	2.0	0.9

Protein kinase A signalling is required for rhabdomyosarcoma tumor maintenance

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(manuscript in preparation)

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Running title: PAX3 target genes

Keywords: embryonal RMS / oncogenic transcription factor / expression profiling/

Abstract

PAX3 is a transcription factor whose expression is associated with ebryonal rhbdomyosarcoma (eRMS). However, the molecular mechanisms mediating essential pathophysiological functions of PAX3 remain poorly defined. Here, we compared expression profiling of PAX3 silencing in eRMS cells with microarray data avaible from overexpression experiments in different cell lines to identify a minimal set of physiologically important target genes irrespectively of cellular background. Surprisingly, we identified only a single gene to be regulated by PAX3 under all conditions analyzed. This gene, PRKAR2B, was not recognized as PAX3 target before and implicates PKA signalling in mediating PAX3 function. Furthermore our data suggests the involvement of TRAIL-induced apoptosis in eRMS.

Introduction

PAX3 belongs to a family of genes encoding transcription factors. To date, nine members (PAX1-PAX9) have been described (Dahl et al., 1997; Stuart and Gruss, 1996) which are involved in regulation of tissue development and cellular differentiation in embryos. PAX3 plays a pivotal role during neural tube closure (Stuart and Gruss, 1996) and PAX3 expression is required during embryogenesis for the proper migration and specification of myogenic progenitor cells (Relaix et al., 2004). Different PAX family members have been assigned with a wide range of cancers (Muratovska et al., 2003). The precise role of PAX transcription factors in cancer is not known, but it has been shown that PAX genes are preventing terminal differentiation and maintaining progenitor cell state (Tremblay et al., 1996). Two members of the PAX gene family, PAX3, and PAX7, show tumor-associated expression in different cancer types, among them neuroblastomas (Harris et al., 2002) and squamos cell lung carcinomas (Racz et al., 2000). Furthermore, PAX3 has been shown to contribute to melanoma tumor development (Scholl et al., 2001). In melanoma, the function of PAX3 at a nodal point in adult melanocyte stem cell differentiation has been described, where PAX3 simultaneously initiates melanogenic cascade while preventing terminal differentiation (Lang et al., 2005). Also in different sarcoma types such as rhabdomyosarcoma and Ewing's sarcoma constitutive PAX3 and PAX7 expression has been reported (Barr et al., 1999).

There are two major subtypes of rhabdomyosarcoma, the alveolar (aRMS) and embryonal rhabdomyosarcoma (eRMS). In both subtypes, PAX3 and PAX7 are thought to be involved in tumorigenesis. In 80% of the aRMS subtype an abberant transcription factor is generated by the reciprocal translocation t(2;13)(q35;q14) or t(1;13)(p36;q14), which leads to the fusion of PAX3 or PAX7 to FKHR, also known as FOXO1A (Davis et al., 1995; Galili et al., 1993). In PAX3(7)/FKHR, the two DNA binding domains, namely paired and homeodomain, derive from PAX3(7) and the stronger transactivation domain from FKHR. As PAX3(7) derived DNA binding domain is identical in PAX3(7) and PAX3(7)/FKHR encoded proteins, this leads to the assumption that these transcription factors would target similar genes and that PAX3(7)/FKHR acts as stronger transactivator than the wild-type PAX3(7), leading to upregulation of PAX3(7) target genes. This conclusion was supported by recent microarrays studies describing translocation-dependent gene signature (Davicioni et al., 2006; Wachtel et al., 2004). Moreover, TFAP2beta, one of the best discriminating genes between eRMS and aRMS detected by microarray analysis, was recently revealed as a downstream-target of PAX3/FKHR and is involved in PAX3/FKHR mediated tumor-maintenance (submitted manuscript). Furthermore, in vivo data support the hypothesis of common genes targeted by wild-type and fusion protein, as the phenotype of PAX3-mutant mice could be rescued by introducing of a PAX3/FKHR gene (Relaix et al., 2003).

ERMS is not associated with specific translocation, however, PAX3 is implicated in the invasive and metastatic potential of eRMS. Recent findings show that it might do so by maintenance of a progenitor cell state and promoting cell survival. This finding was supported by several studies demonstrating the "oncogene –addiction" of eRMS tumors, as silencing of PAX3 gene by siRNA or oligonucleotides results in induction of apoptosis (submitted manuscript)(Bernasconi et al., 1996; Elbashir et al., 2001).

On the other hand, there are data suggesting that PAX3 and PAX3/FKHR have slightly different

binding properties and thus are promoting the expression of different target genes, depending on cellular background and differentiation status of cells (Begum et al., 2005; Linardic et al., 2005). To date several target genes of PAX3 and PAX3/FKHR which can activate the myogenic transcription program have been reported (Khan et al., 1999). Several PAX3/FKHR targets, among them MYCN (Khan et al., 1999) or bcl-xl have been identified (Margue et al., 2000). The best characterized gene is c-met (Relaix et al., 2003), whose PAX3-dependent expression in muscle precursor cells is essential for proper migration. Recent findings however suggest that precursor cell may be different in eRMS and aRMS (Linardic et al., 2005); while the specificity of PAX3/FKHR fusion gene for aRMS suggests that the cell context is critical for oncogenesis, the parental alveolar rhabdomyosarcoma cell of origin remains unknown. Consistent with this finding, recent microarray analysis could reveal that PAX3/FKHR alters the expression of gene targets in a cell-type specific manner quantitatively and qualitatively distinct from PAX3 (Begum et al., 2005). As anticipated, a PAX3/FKHR specific gene signature could not be detected in eRMS.

Numerous publications recently performed microarray approaches in order to identify novel PAX3/FKHR and PAX3 target genes. A PAX3/FKHR specific gene signature which defines a group of potential PAX3/FKHR target genes was revealed using biopsy samples (Davicioni et al., 2006; Wachtel et al., 2004). For identification of PAX3 targets, overexpression studies have been performed in medulloblastoma and osteosarcoma cells and subsequently combined with microarray analysis (Begum et al., 2005; Mayanil et al., 2001). Furthermore, similar approaches have been used with PAX3/FKHR which was overexpressed in RD (Davicioni et al., 2006), NIH3T3 (Khan et al., 1999) and osteosarcoma cells (Begum et al., 2005) and the resulting changes detected by microarray analysis have been described. Moreover, a similar approach was also performed with the murine RMS cells 76-9 (Nabarro et al., 2005).

However, since the results obtained in these studies are not consistent, we hypothesized that overexpression in different cellular backgrounds contributes to broad variation in data. To avoid complications by cell background, we choose a complementary approach for identification of PAX3 target genes and combined siRNA-mediated down-regulation strategy in eRMS cells with microarray analysis, thus identifying endogenous putative PAX3 targets in eRMS itself. Thereafter, we integrate all available literature data to identify a minimal set of target genes depending on PAX3 irrespective of cellular background.

Material and Methods

Cell lines

Rh4 alveolar rhabdomyosarcoma (aRMS) cells, originating from acites fluid of a St Jude patient at autopsy in 1982, was provided by Peter Houghton (St. Jude Children's Research Hospital, Memphis, TN, USA). RD embryonal rhabdomyosarcoma (eRMS) and 293T human embryonic kidney cell lines were obtained from ATCC (LGC Promochem, Molsheim Cedex, France).
Cells were maintained under proliferating conditions in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and grown in 95% air, 5% CO2 at 37°C.

siRNA-mediated silencing

PAX3 and PAX3/FKHR knockdown was performed with the RNA interference (RNAi) technique (Elbashir *et al*., 2001). A total of $2x10^5$ rhabdomyosarcoma or melanoma cells was plated and 24h later transfected with a combination of two chemically synthesized siRNAs (5'AAGAGAGAACCCGGGCAUG-dTdT and 5'CAUGGAUUUUCCAGCUAUA-dTdT) both targeting the PAX3 part of the fusion gene (Qiagen, Hombrechtikon, Switzerland). Transfection was performed according to manufacture's instructions using 7μl of GeneEraser (Stratagene, La Jolla, CA) and 20nM siRNA (final concentration).

Quantitative RT-PCR

Total RNA samples (1μg) were reverse-transcribed with $Oligo(dT)_{15}$ Primer using the Omniscript Reverse Transcription Kit (Qiagen). Quantitative RT-PCR detection of PAX3 and GAPDH was performed with commercially avaible assays-on-demand Hs00240950_m1, and Hs99999905_m1 (Applied Biosystems, Rotkreuz, Switzerland), respectively. Furthermore, genes involved in apoptotic TRAIL pathway, bcl-2 and one of receptors of TRAIL, TRAILR4, were analysed using commercially avaible assays-on-demand (Hs00236329_m1 and Hs00174664_m1, respectively). TaqMan analysis was carried out according to the manufacturer's instructions on an Applied Biosystems 7900HT Sequence Detector. Expression levels of the gene of interest were normalized with GAPDH expression levels. Experiments were performed in triplicate and standard deviations were calculated based on the results of three biological replicates.

Western Blot

For Western Blot analysis of siRNA mediated effects on protein level, $1x10^7$ cells per treatment were harvested at each indicated time point and nuclear extract was prepared. Protein concentration was measured with the Bradford method using Protein Assay Dye Reagent Concentrate (Bio-Rad, Reinach, Switzerland). 10μg of total protein was then used for western blotting. For PAX3 detection a goat-anti-PAX3(7) antibody (Sigma, Switzerland) was used, mouse-anti-PCNA (Bioscience) was used as control antibody.

Cell Proliferation assays

Cell proliferation was measured using the MTT assay system (Roche, Rotkreuz, Switzerland). A total of $1x10^4$ RD cells were plated per 96-well and transfected 24h later. The amount of converted MTT reagent was measured at different time points up to 72h later by a multi-detection microplate reader (Bio-Tek Instruments, Inc.) at wavelength 595 nm.

Apoptosis assays

One thousand cells from each experimental condition were assayed for caspase-3 activation using the Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's instructions. Caspase activity was measured at an excitation wavelength of 485nm and an emission wavelength of 516nm by a multi-detection microplate reader (Bio-Tek Instruments, Inc.). Experiments were performed in triplicates.

Gene expression analysis

Global changes in gene expression of RD upon knock down of PAX3 by RNAi were measured using Affymetrix HG-133A GeneChip arrays (Affymetrix Inc., Santa Clara, CA). cRNA target synthesis and experimental procedures for GeneChip hybridization and scanning were carried out according to the "GeneChip eukaryotic small sample target labeling technical note" (Affymetrix, Santa Clara, CA). Expression data of siRNA - or scRNA (control) treated and nontreated cells was analyzed using dChip2004 and GeneSpring7.0 software programs. The identified sets of downregulated genes were compared with published microarray data from other studies.

Pathway analysis

The PANTHER (https://panther.appliedbiosystems.com/) classification system was applied to classify proteins (and their genes) according to families, molecular functions and pathways.

The analysis was performed with the set of genes involved in apoptosis, identified by dchip2004 gene expression analysis program to be up – or down regulated upon siRNA treatment at time points 24hrs, 48hrs and 72hrs. Genes involved in the TRAIL pathway were identified and subsequently analyzed using real-time PCR.

Results

1. PAX3 promotes RD cell survival

To characterize PAX3 target genes relevant for eRMS development and maintenance, we first established a siRNA-mediated down-regulation strategy. Therefore, a combination of two chemically synthezised siRNAs, which led to the strongest specific inhibition of PAX3 expression, was used for silencing of PAX3 in the eRMS cell line RD. PAX3 expression was suppressed in RD already after 24h of treatment by nearly 70% on mRNA level. PAX3 protein levels were also reduced, although delayed, to 40% compared to control-treated samples 48h after treatment (Figure 1A).

To characterize the physiological effects occurring upon siRNA treatments, we next measured proliferation of RD after treatment with PAX3 specific and control siRNAs for 24hrs, 48hrs and 72hrs. Cell growth was found to be inhibited significantly in cells treated with siRNA targeting PAX3, whereas normal proliferation rates were observed in untreated or control (scRNA, anti-GAPDH siRNA, transfection reagent alone) cells (Figure 1 B). Furthermore, assessment of apoptotic activities by measuring active caspase 3/7 showed an approximately 1.7-fold increase in enzymatic activity in cells with silenced PAX3 expression compared to control treated cells (Figure 1 C). Thus, an anti-apoptotic function of PAX3 in eRMS cell lines could clearly be demonstrated. These results are in accordance with earlier findings, demonstrating an anti-apoptotic function of PAX3 in eRMS cells treated with specific antisense oligonucleotides (Bernasconi et al., 1996) and therefore validate our siRNA approach also on the physiological level.

2. Comparison between genes downregulated in Rh4 cells and RD cells after PAX3/FKHR and PAX3 downregulation, respectively

First, we identified genes, specifically downregulated in RD cells upon PAX3 silencing. Towards this end, RD cells were treated with siRNA, RNA isolated at time points 0 hrs, 24hrs, 48hrs and 72hrs followed by determination of their expression profile at each time point. To eliminate unspecific effects caused by the treatment procedure, the expression profiles of cells treated with scRNA (control) and non-treated cells were also determined. The microarray data was analysed using GeneSpring 7.0 software (see Methods). First, gene expression values were normalized per chip and per gene (default normalization) and filtered based on a cross-gene-error-model. Genes downregulated more than 1.5 fold in siRNA treated samples and not affected by control treatment, were filtered and the ratio between siRNA and scRNA treatment was calculated. 338 genes represented by 340 probe sets could be identified in this analysis (for entire list see supplementary data).

To identify those genes which are potentially regulated by both, PAX3 and PAX3/FKHR, a direct comparison between genes downregulated in Rh4 cells and RD cells after 24hrs was performed. First, the “present” and “absent” calls had to be modified for further calculation because genes that are highly expressed in Rh4 cells were expressed at low levels in RD cells. This resulted in “absent” calls in treated, but not in untreated cells. We therefore decided to include all genes even if they were “absent”. The analysis was performed at the shortest time point of treatment (24 hrs) assuming that

direct targets are the first genes downregulated. In Rh4 cells, 1834 genes were identified to be specifically down-regulated (>1.5 fold) by siRNA-treatment at time point 24hrs when compared to scRNA treatment. Therefore, a large number of 1834 probe sets specifically downregulated in Rh4 cells were searched for possible target genes of PAX3 in RD cells. All expression values were corrected with the scRNA control levels. Interestingly, only 98 genes out of 1834 were specifically downregulated in same manner in RD cells (>1.5 fold downregulation when compared to control treatment), meaning that almost one third (29%) of PAX3 dependent genes are also regulated in the same manner by PAX3/FKHR, but only 5% of PAX3/FKHR regulated genes are equally regulated by PAX3 (Figure 2). This difference could depend on the characteristic of PAX3/FKHR which is a stronger transactivator than PAX3, therefore more changes of regulated genes can be detected at the same cut-off level. To further constrict relevant PAX3 and PAX3/FKHR target genes, we next performed gene ontology studies. The largest number of genes are involved in signal transduction (32%) among them protein kinases and phosphatases like PRKAR2B (protein kinase, cAMP-dependent, regulatory, type II, beta) or PPP4R2 (protein phosphatase 4, regulatory subunit 2). The second largest number of genes encode for enzymes (18%) and finally, numerous genes (16%) are involved in transcriptional regulation and DNA or protein binding, respective gene names and downregulation levels are indicated in Table 1. These data lead to the conclusion that distinct genes are regulated by PAX3 and PAX3/FKHR, however, also a large overlap could be found between these genes.

3. Comparison with PAX3 targets from PAX3 overexpressing cells

We next sought to compare our list of target genes with the set of previously published putative PAX3 targets. Gene lists from two studies performed to identify PAX3 target genes were compared with our data set. Of the 28 genes identified in osteosarcoma cell line, SaOs (Begum et al., 2005) two genes, PRKAR2B and SEMA3 and, could be identified by the comparison with RD (338) specific genes and one of them, PRKAR2B, was also in common with RD and Rh4 (98) specific genes (Figure 3). Exact gene names are given in Table 2 and Table 3. However, genes encoding for the same family of proteins, like metalloproteinases and zink-finger proteins, could be found to be regulated in both cell types, induced after overexpression in osteosarcoma cells and downregulated by low PAX3 levels in eRMS cells.

The second study revealed 102 genes induced by PAX3 after overexpression in medulloblastoma cells (Mayanil et al., 2001). However, the comparison of this gene list with our data set did not result in an overlap of any genes, suggesting that PAX3 regulated genes are entirely different in medulloblastoma and eRMS cells.

4. Comparison with PAX3 targets from PAX3/FKHR overexpressing cells

PAX3/FKHR has been shown to be a stronger transactivator than PAX3 (Fredericks et al., 1995), therefore several studies have been performed introducing PAX3/FKHR in several cell types and analyzing the resultant gene expression changes, thus expecting more pronounced activation of PAX3 targets by PAX3/FKHR. We therefore hypothesised that genes, upregulated in cells upon ectopic

PAX3(7)/FKHR expression might be common with those which are downregulated upon PAX3 silencing. Three studies performed with NIH3T3, osteosarcoma and RD cells identified different putative target genes induced by PAX3(7)/FKHR overexpresion.

In the first study, PAX3/FKHR was introduced into NIH3T3 cells and a set of 11 genes has been identified by cDNA microarray analysis to be induced specifically by PAX3/FKHR (Khan et al., 1999). However, no overlap could be found with our data set, probably also due to different array techniques.

In a second study, eRMS cell line RD was analysed upon ectopic expression of PAX3/FKHR or PAX7/FKHR and subsequent microarray analysis of PAX3/FKHR, PAX7/FKHR or vector transduced RD population was performed (Davicioni et al., 2006). Thus, 389 genes could be identified as differentially expressed. Comparison with our data sets revealed 9 genes regulated equally by PAX3(7)/FKHR overexpression or PAX3 silencing in RD cells and 4 genes when compared to the smaller set of 98 genes (Figure 4A).

In the third study, PAX3/FKHR overexpression in osteosarcoma cells specifically induced 65 genes (Begum et al., 2005). Comparison of the 65 genes with our data set revealed an overlap of 4 genes when compared to our data (Figure 4B). Interestingly, one gene, PRKAR2B (protein kinase, cAMP-dependent, regulatory, type II, beta), appears to be regulated by PAX3 and PAX3/FKHR in all the studies. All gene names and probe numbers are summarized in Tables 2 and 3.

5. Comparison between genes downregulated in RD cells after PAX3 downregulation and in-vivo data (biopsies)

Next we asked whether any of the 98 genes, regulated by both PAX3 and PAX3/FKHR, might be associated with PAX3 downregulation in vivo. Therefore a comparison between genes, downregulated in RD cells after PAX3 silencing and in vivo data obtained by expression analysis of biopsy samples was performed. To date, there are two main gene signatures discriminating between translocation-positive aRMS and translocation-negative aRMS and eRMS published (Davicioni et al., 2006; Wachtel et al., 2004).

First, the signature list consisting of 417 genes (Wachtel et al., 2004) was compared to the set of 338 genes, downregualted in RD cells and to the set of 98 genes, downregulated in RD and Rh4 cells and resulted in an overlap of 12 and 5 genes, respectively (Figure 5, Table 2 and 3).

In the second study also a gene signature consisting of 534 genes was identified, in this approach a larger data set of 139 biopsies was used (Davicioni et al., 2006). A comparison with our 98 gene set revealed overlap of nine genes (Figure 5). With our 338 gene set, 19 genes were in common. The complete list of overlapping genes is summarized in Table 2 and 3.

Moreover, a subset of 51 direct PAX3/FKHR in vivo target genes identified in Rh4 cells was identified in our previous study (manuscript submitted). Three genes, TFAP2beta, DZIP3 and FGFR2 were downregulated in both sets in RD and Rh4 cells (data not shown).

6. PAX3-dependent pathways

Ontology studies of genes affected by PAX3 downregulation showed different groups of genes according to their function. In the preceding analysis, we searched for direct target genes of PAX3 24 hrs after transfection. Assuming that genes up or downregulated after longer period of time are not direct target genes of PAX3 but probably regulated indirectly, we now also examined gene expression pattern at later time points. Therefore, genes specifically up- or downregulated upon PAX3 silencing were examined at time points 24h, 48h and 72h post-transfection. Unlike in Rh4 cells, no induction of muscle development related genes was observed in RD cells.

A group of apoptosis-related genes regulated indirectly could be identified. Pathway analysis revealed four genes which showed altered expression upon PAX3 silencing, TRAIL-R, Bcl-2, Apaf-1 and caspase 7 were involved in TNF-related apoptosis-inducing ligand (TRAIL) pathway (Figure 6A), which is sufficient to be considered as statistically significant. Therefore, one possible way how apoptosis is mediated in RD cells, could be via TRAIL pathway. Interestingly, one of the genes involved in this pathway, bcl-xl, has already been shown to be transcriptionally activated by PAX3 and PAX3/FKHR (Margue et al., 2000), indicating at least one mechanism of the anti-apoptotic effect of PAX3. For validation of the microarray data, two genes, tumor necrosis factor receptor superfamily, member 10d (TRAIL-R) and blc-2 were also analysed using qRT-PCR (data not shown). Indeed, mRNA expression values of TRAIL-R increased 2.7 fold 24h after PAX3 downregulation as compared to non-silencing scRNA treatment.

Discussion

Understanding of the oncogenic properties of PAX3 requires identification of the relevant downstream targets. We demonstrated previously that a large set of PAX3/FKHR target genes could be identified by comparative expression analysis of a silencing approach with subsequent microarray analysis and a gene signature identified in tumor biopsies (manuscript submitted). Performing a similar approach, a set of possible target genes was identified using microarray analysis of RD cells after silencing of PAX3.

Using this approach, 98 genes could be found downregulated equally in eRMS and aRMS subtypes by silencing of PAX3 and PAX3/FKHR, respectively. There are different explanations for this result; first, eRMS and aRMS represent two different histological subtypes of RMS with distinct molecular profiles, histology and clinical outcome. While the aRMS subtype is in most cases associated with a specific translocation, no consistent chromosomal rearrangements have been identified in eRMS so far. However, gains and losses of whole chromosomes have been described in eRMS (Bridge et al., 2000; Weber-Hall et al., 1996) and frequently loss of heterozygocity at the 11p15 locus could be detected. Moreover, alterations of genomic imprinting in this locus could be identified (Scrable et al., 1989). The frequent loss of heterozygocity and involvement of genomic imprinting suggest that inactivation of a tumour suppressor gene located in this region could contribute to eRMS tumourgenesis, as both copies of a gene could be inactivated sequentially.

Secondly, PAX3 on its own is a less potent transactivator then PAX3/FKHR; therefore the expression levels of its target genes don't necessarily have to be altered in a prominent way as can be observed in translocation-positive aRMS. Indeed, PAX3/FKHR dependent gene signatures could be identified by overexpression studies in heterologues systems such as osteosarcoma cell line, SaOS-2 (Begum et al, 2005) and cell lines remodelling eRMS, RD (Begum et al, 2005; Davicioni et al, 2006). Such an approach of ectopically expressed PAX3 in medulloblastoma cell line, DAOY, revealed set of genes, altered in their expression by stronger PAX3 expression as compared to endogenous levels (Mayanil et al., 2006). However, in this approach no difference between direct target genes and genes regulated indirectly could be determined. Moreover, not all published microarray data could be compared with our data set, as data obtained with different array techniques (Khan et al., 1999) and data from mouse cell line (Nabarro et al., 2005) could not be incorporated into the comparison.

Third, although RMS is thought to originate from myogenic precursor cells, the precise cell type from which the RMS subtypes originate is still a matter of debate. Recent findings support the hypothesis that the cell of origin in eRMS and aRMS might be different, as introduction of same panel of genetic changes leading to rhabdomyosarcoma in more or less differentiated human skeletal muscle cell of origin gave rise to different tumor morphologies (Linardic et al., 2005). In relation to that, differentiation status might be different in eRMS and aRMS cell of origin, as no induction of differentiation-associated genes could be detected in RD cells, but in Rh4 cells. Consistent with these findings, cell-type-specific regulation of target genes by PAX3 and PAX3/FKHR is possible and was supported by findings of Begum et al., revealing a distinct but overlapping set of genes targeted by PAX3/FKHR and PAX3 in a cell-type specific manner.

For our analytical set up to identify relevant PAX3 target genes, in vivo data is crucial; therefore a gene signature specifically associated with PAX3 expression in eRMS biopsy samples woeld be desirable. To our knowledge, no gene signature from eRMS biopsy samples which depends exclusively on PAX3 expression has been published so far. Taking into account that PAX3 expression is required for cancer cell survival in eRMS (Bernasconi et al, 1996) and - suggested by our results and confirmed in previous studies - in melanoma cells (Scholl et al, 2001; Muratovska et al, 2003), and most eRMS cell line show increased PAX3 expression (Bernasconi et al, 1996; Barr et al, 1999) it can be assumed that the PAX3 expression plays a significant role in oncogenic processes in eRMS.

One gene identified by our comparison approach appears to be regulated in osteosarcoma cells, RD cells and RMS biopsy samples by both, PAX3 and PAX3/FKHR is PRKAR2B, a cAMP dependent protein. This was an unexpected result, as no other known target of PAX3 or PAX3/FKHR appeared to be regulated in cell independent manner. Moreover, no implication of PRKAR2B in eRMS development has been described so far. As shown recently, protein kinase A targets the transcription factor CREB and plays a role in the induction of myogenic determination genes such as PAX3, MyoD and Myf5 via Wnt signaling (Chen et al., 2005). However, direct activation of PAX3 by CREB is possible, as core CRE sites are present in the promoter region of PAX3. Thus, physiologic interaction between PAX3 and PRKAR2B could contribute to signal transduction impeding myogenic differentiation and thus contribute to tumorigenesis in eRMS. However, although microarray data from several independent studies suggest PAX3 dependent regulation of PRKAR2B in different cell types and its contribution to cancer formation as a signal molecule is likely, no further studies of PRKAP2B role in eRMS are available to date. We therefore aim to reveal the role of PRKAR2B in our eRMS cell models, which is the subject of ongoing work.

It has been shown in different studies including this one, that PAX3 plays a role in apoptosis and cell survival. Although the anti-apoptotic protein bcl-xl has been described to be regulated by PAX3 (Margue et al., 2000), little is known about pathways mediating the anti-apoptotic properties of PAX3. One possible pathway how apoptosis is mediated in RD could be determined with our microarray study, namely the TNF-related apoptosis-inducing ligand (TRAIL) pathway. TRAIL induced apoptosis could be an important aspect in clinical treatment since there are numerous studies describing TRAIL resistance in different sarcoma types (Komdeur et al., 2004). Indeed, TRAIL-sensitive cell lines of both embryonal and alveolar histology have been described (Petak et al., 2000). RD cells were found to be highly sensitive to TRAIL pathway in this study, thus supporting our findings. Nevertheless, no involvement of TRAIL pathway in Rh4 cells could be detected by microarray analysis in our approach. Thus, the physiological relevance of our microarray data remains to be proven. However, this approach may be important for development of new therapeutic approaches for the treatment of RMS.

Our approach of combination of RNAi mediated silencing of PAX3 transcription factor with subsequent microarray analysis revealed one possibility for the search of new pathways for better therapeutically relevant targets and treatments. Moreover, our data complies with previous findings supporting the theory that eRMS and aRMS represent distinct entities with likely different origins where besides altered expression of PAX3 or PAX3/FKHR additional molecular alterations contribute to tumorgenesis.

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Figure legends

Figure 1 Physiological effects of PAX3 silencing in RD cells
(A) mRNA and protein levels of PAX3 as measured by qRT-PCR analysis and Western blot after RNAi mediated downregulation in eRMS cell RD. For mRNA levels, the ratio of siRNA to scRNA (control) treatment is shown in %. In the densiometric analysis of protein amount the siRNA and scRNA (control) treated samples were normalized with PCNA levels and the ratio calculated (B) Proliferation of RD cells as measured by MTT assay after 72h of treatment with either PAX3 specific siRNA, scRNA, siRNA targeting GAPDH, or transfection reagent alone (GeneEraser). The means ±standard deviations (error bars) from three independent triplica experiments are shown. (C)) Induction of apoptosis in RD cells after the indicated time periods of treatment with either PAX3 specific siRNA, scRNA, siRNA targeting GAPDH or GeneEraser (transfection reagent) as measured by caspase-3/7 assay. Fold change values of caspase activity of treated vs. non-treated cells from two independent triplica experiments are shown.

Figure 2 Changes in gene expression after PAX3 and PAX3/FKHR silencing in eRMS and aRMS cells
(A) Diagram depicting normalized expression values (log10 scale) of 1834 specifically downregulated genes as measured in Rh4 cells 24h after PAX3/FKHR silencing. Global expression changes were measured in Rh4 cells after 0, 24, 48 and 72hrs of treatment with PAX3-specific siRNA. Each line represents the expression of one gene. (B) Blot depicting the sam set of genes of control-treated Rh4 cells (scRNA). (C) Diagram depicting normalized expression values (log10 scale) of 340 specifically downregulated genes as measured in RD cells 0, 24h, 48 and 72hrs after PAX3 silencing. (D) Expression pattern of control-treated Rh4 cells (scRNA) as in C. (E) Diagram depicting the overlap between PAX3/FKHR-specific downregulated genes identified in Rh4 cells and genes downregulated after PAX3-specific siRNA treatment. 98 genes are present in both sets of genes. (F) Graphic representation depicting the ontology of the 98 potential PAX3 and PAX3/FKHR target genes identified in E. A complete list of the functions as well as downregulation levels of the 98 genes in RD and Rh4 cells is shown in Tab. 1.

Figure 3 Comparison of genes downregulated in RD after PAX3 silencing and induced by PAX3 overexpression in osteosarcoma cells
Venn diagram representing the overlap between 338 genes specifically downregulated in RD cells, 98 genes specifically downregulated in RD and Rh4 cells and published set of 28 genes induced by overexpression of PAX3 in osteosarcoma cells (Begum et al., 2005). All gene names identified by the overlap are listed in Table 2 and 3.

Figure 4 Comparison of genes downregulated in RD after PAX3 silencing and induced by PAX3/FKHR overexpression in osteosarcoma and RD cells
(A) Venn diagram representing the overlap between 338 genes specifically downregulated in RD cells, 98 genes specifically downregulated in RD and Rh4 cells and published set of 389 genes induced by overexpression of PAX3/FKHR in RD cells (Davicioni et al., 2006). All gene names identified by the

overlap are listed in Table 2 and 3. (B) Venn diagram representing the overlap between 338 genes specifically downregulated in RD cells, 98 genes specifically downregulated in RD and Rh4 cells and published set of 65 genes induced by overexpression of PAX3/FKHR in osteosarcoma cells (Begum et al., 2005). All gene names identified by the overlap are listed in Table 2 and 3.

Figure 5 Comparison of RD cells gene expression changes and biopsy data
(A) Venn diagram depicting the overlap between a PAX3(7)/FKHR-specific gene signature consisting of 417 genes resp. 534 genes derived from RMS biopsies and 98 genes downregulated by PAX3-specific siRNA treatment in both, RD and Rh4 cells (B) Venn diagram depicting the overlap between a PAX3(7)/FKHR-specific gene signature consisting of 417 genes resp. 534 genes derived from RMS biopsies and 338 genes downregulated by PAX3-specific siRNA treatment in RD cells

Figure 6 TRAIL pathway involved in PAX3 mediated apoptosis
(A) Scheme of TRAIL pathway is shown, apoptosis-related genes identified by microrray analysis are highlighted in red.

Figure 1

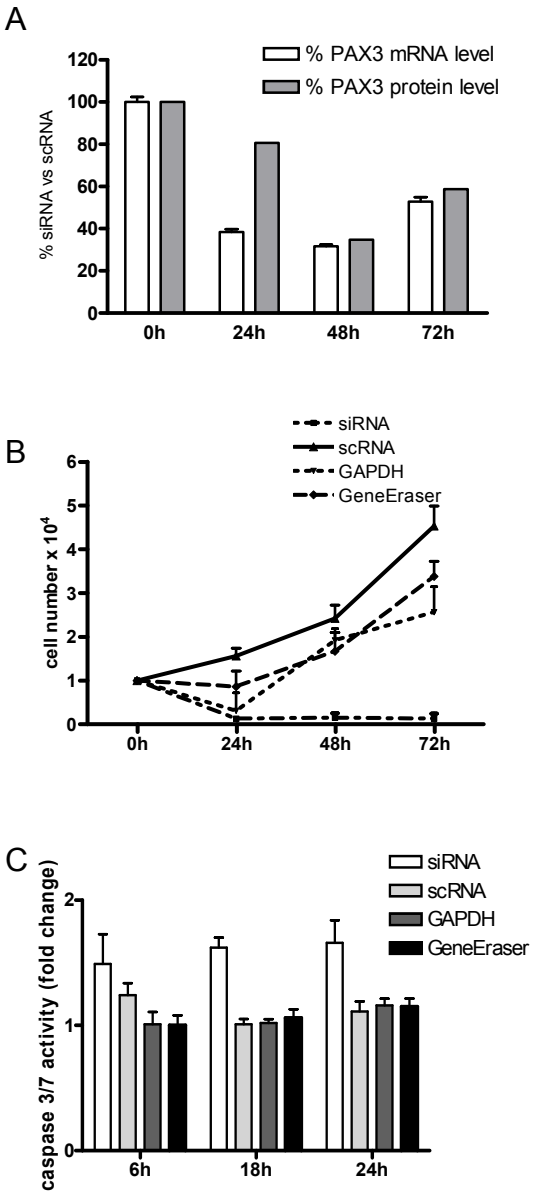
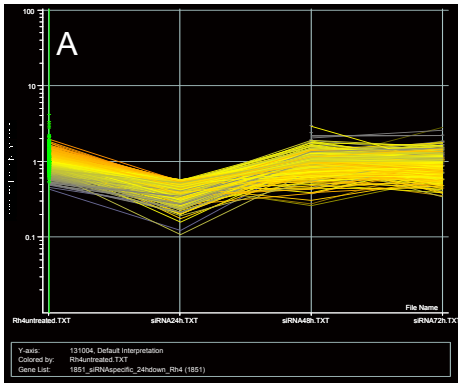
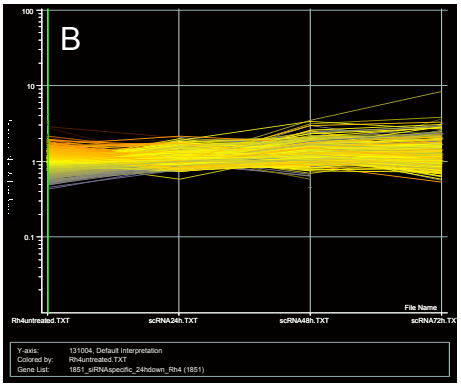

A
% PAX3 mRNA level
% PAX3 protein level
% siRNA vs scRNA
120
100
80
60
40
20
0
0h
24h
48h
72h
B
siRNA
scRNA
GAPDH
GeneEraser
cell number x 10^4
6
5
4
3
2
1
0
0h
24h
48h
72h
C
siRNA
scRNA
GAPDH
GeneEraser
caspase 3/7 activity (fold change)
2
1
0
6h
18h
24h

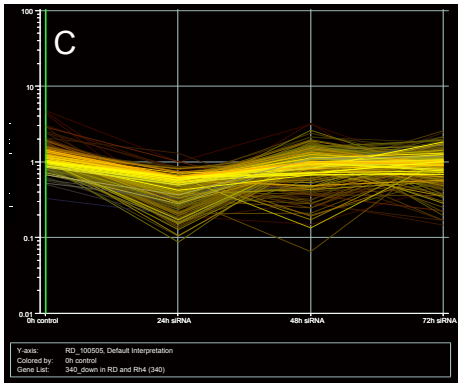
Figure 2



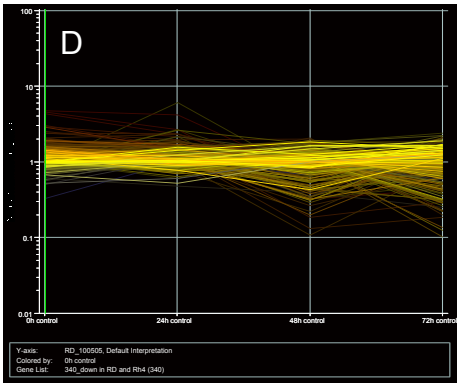
Rh4_siRNA



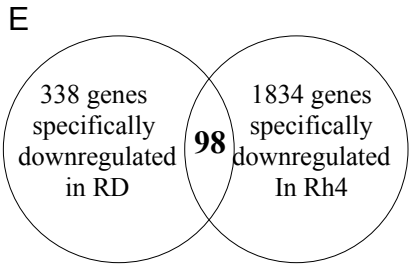
Rh4_scRNA



RD_siRNA



RD_scRNA



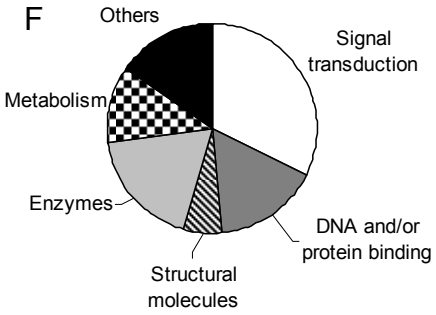
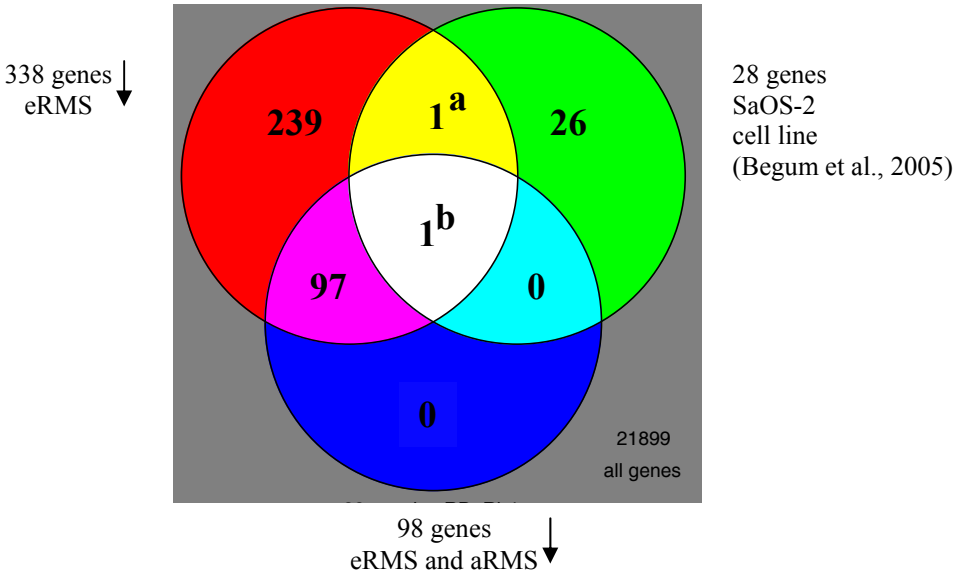


Figure 3

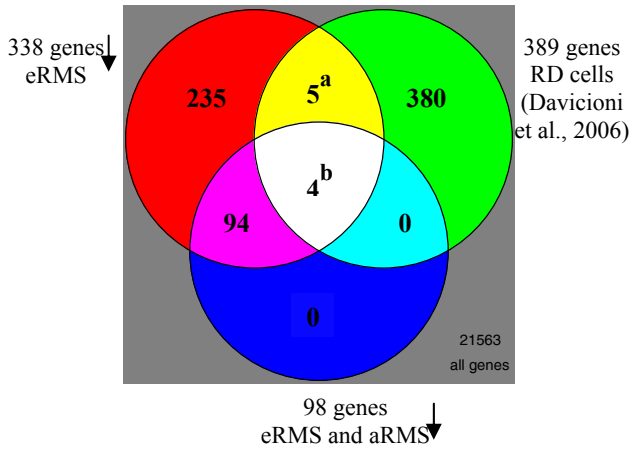


a SEMA3 sema domain, secreted, (semaphorin) 3C

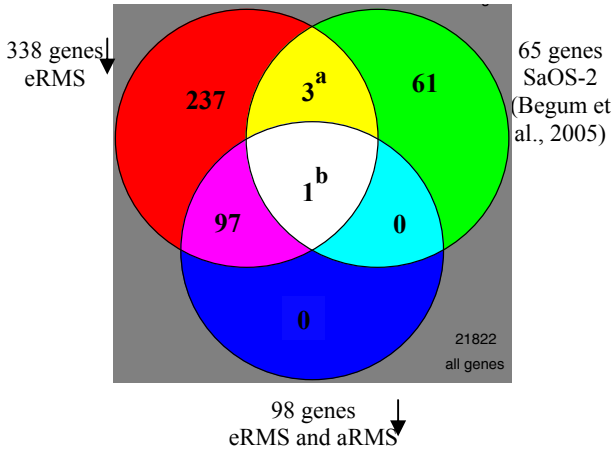
b PRKAR2B protein kinase, cAMP-dependent, regulatory, type II, beta

Figure 4

A



B



a

PAX5	paired box gene 5 (B-cell linage specific activator protein)
MAGEL2	MAGE-like 2
LRP5	low density lipoprotein receptor-related protein 5
SCN3A	sodium channel, voltage-gated, type III, alpha
STB1	special AT-richsequence binding protein 1 (binds to nuclear matrix/scaffold-associating DNA's

b

CMKOR1	chemokine orphan receptor 1
STATH	statherin
LDOC1	leucine zipper, down-regulated in cancer
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta

a

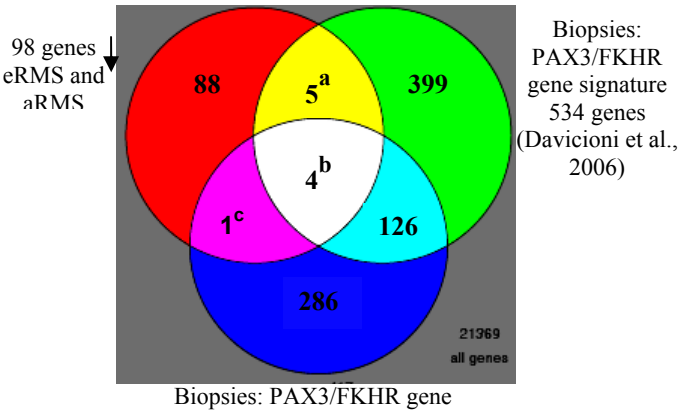
CALCRL	calcitonin receptor-like
SCAMP1	secretory carrier membrane protein 1
SEMA3C	sema domain, secreted, (semaphorin) 3C

b

PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta

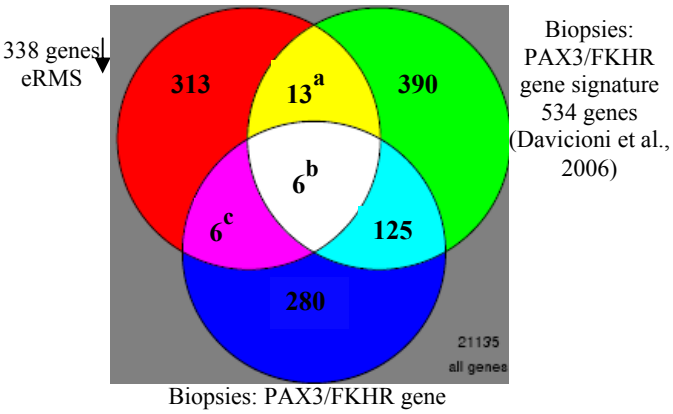
Figure 5

A



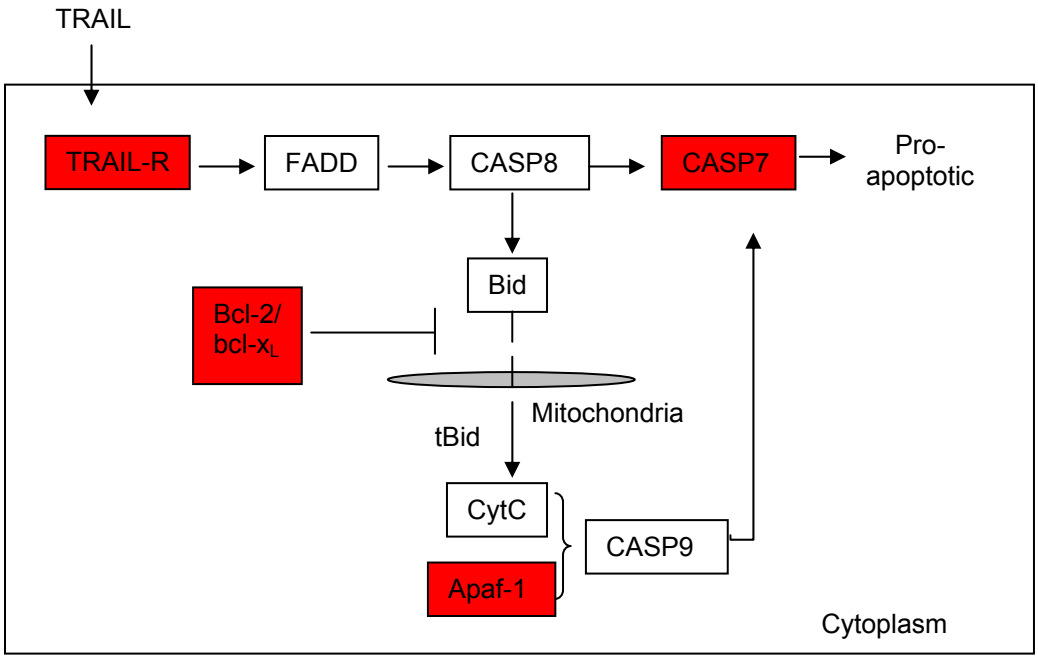
a	PRKAP2B	protein kinase, cAMP-dependent, regulatory, type II, beta
	GPC4	glypican 4
	PEG3	paternally expressed 3
	TNRC9	trinucleotide repeat containing 9
	TSC22D2	TSC22 domain family 2
b	TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta) from Pol II promoter
	DZIP3	zinc finger DAZ interacting protein 3
	EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2
	FGFR2	keratinocyte growth factor receptor 2
c	LDOC1	leucine zipper, down-regulated in cancer

B



a	PAX5	paired box gene 5
	MAGEL2	MAGE-like 2
	KIAA0657	KIAA0657 protein
	SC65	synaptonemal complex protein SC65
	PRKX	protein kinase, X-linked
	PRKAP2B	protein kinase, cAMP-dependent, regulatory, typeII, beta
	GPC4	glypican 4
	HBLD2	HESB like domain containing 2
	PEG3	paternally expressed 3
	PTP4A3	protein tyrosine phosphatase type IVA, member 3
	RRBP1	ribosome binding protein 1 homolog 180kDa (dog)
	SCAMP1	secretory carrier membrane protein 1
	TNRC9	trinucleotide repeat containing 9
b	KIAA0523	KIAA0523 protein
	TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta) from Pol II promoter
	EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor,2
	DZIP3	zinc finger DAZ interacting protein 3
	FGFR2	keratinocyte growth factor receptor 2
	TBCD	tubulin-specific chaperone d
c	UBE2G2	ubiquitin-conjugating enzyme E2G 2 (UBC7 homlog, yeast)
	TU3A	TU3A protein
	LDOC1	leucine zipper, down-regulated in cancer 1
	KIAA0669	KIAA0669 gene product
	NEU3	sialidase 3 (membrane sialidase)
	NUDT4	nudix (nucleoside diphosphate linked moiety X)-type motif 4

Figure 6


TRAIL
TRAIL-R
FADD
CASP8
CASP7
Pro-
apoptotic
Bid
Bcl-2/
bcl-xL
Mitochondria
tBid
CytC
CASP9
Apaf-1
Cytoplasm

Tab. 1 Ontology and downregulation levels of 98 PAX3 and PAX3/FKHR regulated genes

Gene Name	Common	Description	RD fold down	Rh4 fold down
Signal transduction				
204966_at	BAI2	brain-specific angiogenesis inhibitor 2	1.5	2.1
209236_at	SLC23A1	Contains the 3' end of the SLC23A1 gene encoding solute carrier family 23 (nucleobase transporters) member 1	1.5	3.9
207990_x_at	ACRV1	acrosomal vesicle protein 1	1.5	4
209392_at	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	1.6	3.6
213305_s_at	PPP2R5C	protein phosphatase 2, regulatory subunit B (B56), gamma isoform	1.6	6.4
219744_at	FN3K	fructosamine-3-kinase	1.6	3
204037_at	EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	1.7	3.6
201602_s_at	PPP1R12A	protein phosphatase 1, regulatory (inhibitor) subunit 12A	1.7	8.7
221372_s_at	P2RX2	purinergic receptor P2X, ligand-gated ion channel, 2	1.8	2.9
208376_at	CCR4; CKR4; k5-5; CMKBR4; ChemR13; CC-CKR-4	chemokine (C-C) receptor 4; Homo sapiens chemokine (C-C motif) receptor 4 (CCR4), mRNA	1.9	4
203126_at	IMPA2	inositol(myo)-1(or 4)-monophosphatase 2	1.9	1.5
206084_at	PTPRR	protein tyrosine phosphatase, receptor type, R	2.0	2
215524_x_at	Hs.495215	T-cell antigen receptor alpha (TCRA)	2.1	7.5
218180_s_at	EPS8L2	EPS8-like 2	3.1	5.1
203680_at	PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta	3.9	3.8
220764_at	PPP4R2	protein phosphatase 4, regulatory subunit 2	3.9	3.7
206249_at	MAP3K13	mitogen-activated protein kinase kinase kinase 13	4.0	6.1
212977_at	CMKOR1	chemokine orphan receptor 1	7.3	3.1
209697_at	PPP3CC	protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform (calcineurin A gamma)	9.1	2.8
209795_at	CD69	CD69 antigen (p60, early T-cell activation antigen)	10.4	3.6
DNA and/or protein binding				
220748_s_at	ZNF580	zinc finger protein 580	1.5	1.6
207605_x_at	H-plk	Krueppel-related zinc finger protein	1.6	7.7
213473_at	BRAP	BRCA1 associated protein	1.6	2.3
220292_at	ZNF434	synonyms: MGC4179, FLJ20417, FLJ31901; Homo sapiens zinc finger protein 434 (ZNF434), mRNA	1.7	9.1
202600_s_at	NRIP1	nuclear receptor interacting protein 1	1.7	1.9
208003_s_at	NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	1.8	1.6
210954_s_at	TSC22D2	TSC22 domain family 2	1.9	19.7
214774_x_at	TNRC9	trinucleotide repeat containing 9	2.6	5.2
222026_at	RBM3	RNA binding motif (RNP1, RRM) protein 3	2.7	2.7
202617_s_at	MECP2	methyl CpG binding protein 2 (Rett syndrome)	2.9	3.1
214447_at	ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	3.4	3.6
209870_s_at	APBA2	amyloid beta (A4) precursor protein-binding, family A, member 2 (X11-like)	3.5	2.3
213068_at	DPT	dermatopontin	4.2	3.1
214451_at	TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta)	8.9	2.6
209243_s_at	PEG3	paternally expressed 3	11.9	2.9
204907_s_at	BCL3	B-cell CLL/lymphoma 3	15.7	2.7
Structural molecules				
206835_at	STATH	statherin	1.5	2.2
203045_at	NINJ1	ninjurin 1	1.6	2
208623_s_at	VIL2	villin 2 (ezrin)	1.6	3.5
207146_at	KRTHA2	keratin, hair, acidic, 2	1.9	2.5

206509_at	PIP	prolactin-induced protein	2.3	3.8
203352_at	ORC4L	origin recognition complex, subunit 4-like (yeast)	3.3	2.8
Enzymes				
215867_x_at	CA12	carbonic anhydrase XII	1.5	3.4
213540_at	HSD17B8	hydroxysteroid (17-beta) dehydrogenase 8	1.5	3.7
220641_at	NOX5	NADPH oxidase, EF hand calcium-binding domain 5	1.5	2.6
213991_s_at	HS3ST1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	1.5	4.6
219113_x_at	DHRS10	dehydrogenase/reductase (SDR family) member 10	1.5	6.7
207751_at	DPYS	dihydropyrimidinase	1.6	3.1
205369_x_at	DBT	dihydrolipoamide branched chain transacylase	1.9	2.4
205778_at	KLK7	kallikrein 7 (chymotryptic, stratum corneum)	1.9	2.1
206867_at	GCKR	glucokinase (hexokinase 4) regulatory protein	2.2	2.2
219028_at	HIPK2	homeodomain interacting protein kinase 2	2.3	2
207316_at	HAS1	hyaluronan synthase 1	2.3	3.1
214627_at	EPX	eosinophil peroxidase	2.4	2.5
213536_s_at	UBE2I	ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)	2.6	3.1
205652_s_at	TTLL1	tubulin tyrosine ligase-like family, member 1	3.0	3.2
213952_s_at	ALOX5	arachidonate 5-lipoxygenase	3.5	3.1
205158_at	RNASE4	ribonuclease, RNase A family, 4	3.9	4.1
209993_at	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	4.0	2.2
201815_s_at	TBC1D5; KIAA0210	Homo sapiens TBC1 domain family, member 5 (TBC1D5), mRNA	4.9	9.3
Metabolism				
215085_x_at	DLEC1	deleted in lung and esophageal cancer 1	1.5	1.7
217556_at	CLCN4	chloride channel 4	1.5	4.4
200866_s_at	PSAP	prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	1.6	2.6
204984_at	GPC4	glypican 4	1.6	5.8
219911_s_at	SLCO4A1	solute carrier organic anion transporter family, member 4A1	1.7	12.5
210440_s_at	CDC14A	CDC14 cell division cycle 14 homolog A (S. cerevisiae)	1.8	3.6
205738_s_at	FABP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	1.8	5.9
210164_at	GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	2.0	2
204454_at	LDOC1	leucine zipper, down-regulated in cancer 1	2.5	2.8
214287_s_at	CDC2L5	cell division cycle 2-like 5 (cholinesterase-related cell division controller)	5.6	4
210240_s_at	CDKN2D	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	8.4	2.1
215047_at	TRIM58	tripartite motif-containing 58	12.8	14.6
Others				
212483_at	IDN3	IDN3 protein	1.5	1.5
221031_s_at	DKFZP434F0318	hypothetical protein DKFZp434F0318	1.5	11.4
219252_s_at	FLJ20514	hypothetical protein FLJ20514	1.6	3.5
214204_at	PACRG	PARK2 co-regulated	2.1	5.1
218591_s_at	FLJ14075	hypothetical protein FLJ14075	2.5	5.6
222325_at	NCRMS	non-coding RNA in rhabdomyosarcoma (RMS)	2.6	3.1
216562_at	SRMP1; dJ1057D4.1	spermidine synthase (SPDSY) pseudogene match: proteins: Sw:O82147 Sw:Q12074 Sw:Q09741 Sw:Q64674;	2.8	4.4
212289_at	ANKRD12	ankyrin repeat domain 12	2.9	3.6
216063_at	HBBP1	hemoglobin, beta pseudogene 1	2.9	2
217252_at	OSIL;A170; p62B	oxidative stress induced like	3.0	2.3
217239_x_at		VH gene family III; VDJ rearrangement; Homo sapiens lymphocyte-predominant Hodgkin's disease case #4 immunoglobulin heavy chain gene, variable region, partial cds	3.0	4.9
221273_s_at	DKFZP761H1710	hypothetical protein DKFZp761H1710	3.5	1.5
217362_x_at	HLA-DRB3	major histocompatibility complex, class II, DR beta 3	3.6	2.8
216163_at	HBLD2	HESB like domain containing 2	4.5	3.8
204047_s_at	C6orf56	chromosome 6 open reading frame 56	4.9	6

Table 2. Comparison of 98 genes, specifically downregulated in RD and Rh4 cells with published data

Gene name	Description
1. Genes upregulated by PAX3 in osteosarcoma cells (Begum et al., 2006)	
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta
2. Genes upregulated by PAX3/FKHR in osteosarcoma cells (Begum et al., 2006)	
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta
3. Genes upregulated in RD upon ectopic expression of PAX3(7)/FKHR (Supplementary Table S3)	
CMKOR1	chemokine orphan receptor 1
STATH	statherin
LDOC1	leucine zipper, down-regulated in cancer
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta
4. in-vivo data obtained from biopsies (Wachtel et. al., 2004)	
TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta) from Pol II promoter
DZIP3	zinc finger DAZ interacting protein 3
LDOC1	leucine zipper, down-regulated in cancer
EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2
FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)
5. Signature identified by Triche (Supplementary Table S2) 534 genes	
TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta) from Pol II promoter
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta
EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2
GPC4	glypican 4
PEG3	paternally expressed 3
DZIP3	zinc finger DAZ interacting protein 3
FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)
TNRC9	trinucleotide repeat containing 9
TSC22D2	TSC22 domain family 2

Tab 3. Comparison of 338 genes, specifically downregulated in RD cells with published data

Gene name	Description
1. Genes upregulated by PAX3 in osteosarcoma cells (Begum et al., 2006)	
SEMA3	sema domain, secreted, (semaphorin) 3C
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta
2. Genes upregulated by PAX3/FKHR in osteosarcoma cells (Begum et al., 2006)	
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta
CALCRL	calcitonin receptor-like
SCAMP1	secretory carrier membrane protein 1
SEMA3C	sema domain, secreted, (semaphorin) 3C
3. Genes upregulated in RD upon ectopic expression of PAX3(7)/FKHR (Supplementary Table S3)	
PAX5	paired box gene 5 (B-cell lineage specific activator protein)
MAGEL2	MAGE-like 2
SCN3A	sodium channel, voltage-gated, type III, alpha
LRP5	low density lipoprotein receptor-related protein 5
LDOC1	leucine zipper, down-regulated in cancer 1
PRKAR2B	Protein kinase, cAMP-dependent, regulatory, type II, beta
CMKOR1	chemokine orphan receptor 1
SATB1	special AT-rich sequence binding protein 1 (binds to nuclear matrix/scaffold-associating DNA's)
STATH	statherin
4. in-vivo data obtained from biopsies (Wachtel et. al., 2004)	
UBE2G2	ubiquitin-conjugating enzyme E2G 2 (UBC7 homolog, yeast)
TU3A	TU3A protein
LDOC1	leucine zipper, down-regulated in cancer 1
EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2
KIAA0523	KIAA0523 protein
TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta) from Pol II promoter
DZIP3	zinc finger DAZ interacting protein 3
FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)
KIAA0669	KIAA0669 gene product
NEU3	sialidase 3 (membrane sialidase)
NUDT4	nudix (nucleoside diphosphate linked moiety X)-type motif 4
TBCD	tubulin-specific chaperone d
5. Signature identified by Triche (Supplementary Table S2) 534 genes	
PAX5	paired box gene 5 (B-cell lineage specific activator protein)
MAGEL2	MAGE-like 2
KIAA0657	KIAA0657 protein
KIAA0523	KIAA0523 protein
SC65	synaptonemal complex protein SC65
PRKX	protein kinase, X-linked
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta
TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta) from Pol II promoter
DZIP3	zinc finger DAZ interacting protein 3
EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2
FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)

GPC4	glypican 4
HBLD2	HESB like domain containing 2
PEG3	paternally expressed 3
PTP4A3	protein tyrosine phosphatase type IVA, member 3
RRBP1	ribosome binding protein 1 homolog 180kDa (dog)
SCAMP1	secretory carrier membrane protein 1
TBCD	tubulin-specific chaperone d
TNRC9	trinucleotide repeat containing 9

A kinase inhibitor influences PAX3/FKHR activity and exerts potent in vivo anti-tumor activity against alveolar rhabdomyosarcoma

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(manuscript submitted)

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Abbreviations: RMS, rhabdomyosarcoma; ARMS, alveolar rhabdomyosarcoma; TF, transcription factor; RTK, receptor tyrosine kinase

Abstract

Constitutive active signaling pathways are a characteristic phenomenon for many tumor types and tumor cells are often addicted to continuous signaling. Specific inhibition of such pathways has been demonstrated to be an effective therapeutic approach in different tumors. So far, mainly upstream components of these pathways have been used as molecular targets, such as growth factor receptors, which contain drugable enzymatic activities. However, in many tumors more downstream components of signaling pathways are mutationally activated, such as in alveolar rhabdomyosarcoma (aRMS), in which specific translocations lead to the formation of the chimaeric transcription factor PAX3/FKHR. Here, we present evidence for a mechanism allowing regulation of PAX3-FKHR activity by the kinase inhibitor PKC412 (N-benzoyl-staurosporine). The drug influences the phosphorylation status of PAX3-FKHR and hence its transcriptional activity. Furthermore, we demonstrate a potent antitumorigenic potential of PKC412 for aRMS *in vitro* and *in vivo*. Our study suggests that targeting of kinases controlling the activity of oncogenic transcription factors by small molecule inhibitors is a promising therapeutic strategy against cancer.

Introduction

Most tumors show molecular aberrations in signal transduction pathways controlling proliferation, apoptosis, angiogenesis and metastasis. In principle, such aberrations can occur at any site of the signal transduction cascade, including transmembrane growth factor receptors at the beginning of a cascade (e.g., EGF-R, VEGF-R, FLT-3), more downstream located cytoplasmic signal transduction kinases (e.g., mTOR, PKC, AKT/PKB) {Blume-Jensen, 2001 #369} {Gschwind, 2004 #370} {Bianco, 2006 #371} or transcription factors (TF) at the end of a cascade directly leading to disregulated gene expression.

Importantly, in many tumor types, tumors cells have been shown to acquire a dependency on these constitutive active signaling cascades. Hence, inhibition of the aberrant constitutive signaling inhibits tumor growth and since the success of Gleevec as a targeted therapy for chronic myeloid leukemia (CML), the principle of specific targeting of aberrant signal transduction pathways by small molecule inhibitors is thought to be a promising method to fight cancer {Manley, 2002 #372} {Levitzki, 2002 #373} which has been expanded to many other tumor types. This approach seems especially suitable for tumors with aberrations in upstream components of signaling cascades, cell surface receptors, or cytoplasmic signal transduction kinases, as the involved proteins are easily accessible for inhibiting antibodies or often contain drugable enzymatic activities. So far, it has not been applied to tumors containing aberrations located at the end of signaling cascades, such as TF, for which fewer, if any, specific small molecule inhibitors are available. However, as TF represent the second most frequently mutated class of proteins in tumors {Futreal, 2004 #307}, the availability of specific inhibitory compounds against this class of proteins would broaden the principle of targeted therapy to many additional tumor types.

Rhabdomyosarcoma (RMS) accounts for 5 – 8 % of all pediatric malignancies and is the most common soft tissue sarcoma diagnosed in children {Merlino, 1999 #374}, and no targeted drug therapy is available

so far. Histopathologically, RMS can be classified into various subtypes with two major forms, alveolar RMS (aRMS) and embryonal RMS (eRMS). 80% of the aRMS are characterised by specific translocations that are absent in eRMS. These translocations always include the N-terminal part of PAX3 or PAX7 which in the majority of cases are fused to the C-terminal part of FKHR generating the chimaeric transcription factors PAX3/FKHR (t(2;13)(q35;q14)) or PAX7/FKHR (t(1;13)(p36;q14)). Furthermore, in single cases NCOA1 {Wachtel, 2004 #257} and AFX {Barr, 2002 #43} have been found as PAX3(7) fusion partners. Overexpression of these translocation products, especially of PAX3-FKHR, is associated with a poor prognosis {Sorensen, 2002 #107}. Hence, the 5-year survival rate of aRMS patients is much lower (30 %) compared to the one of eRMS patients (60 %) {Raney, 2001 #375}, and once metastasizing, aRMS become resistant to conventional chemo- and radiotherapy. Therefore, new therapeutic agents are urgently needed to increase the survival rate of aRMS patients.

Importantly, tumor cell survival of translocation-positive aRMS is dependent on continuous expression of the fusion proteins. Downregulation of PAX3/FKHR by antisense oligonucleotide treatment induces apoptotic cell death in aRMS cell lines {Bernasconi, 1996 #333}. Furthermore, inhibition of PAX3 target genes using a KRAB-PAX3 repressor leads to tumor growth inhibition and apoptosis *in vivo* {Ayyanathan, 2000 #376}. However, the technical complexity of their application *in vivo* prevents a clinical implementation of antisense nucleic acids in the near future. Therefore, the identification of other, clinically applicable, mechanisms for PAX3-FKHR inhibition would be of great interest.

In this study, we present evidence for a mechanism allowing regulation of PAX3/FKHR activity by the kinase inhibitor PKC412 {Fabbro, 2000 #327}. PKC412 is known as a potent inhibitor of several kinases including PKC, Akt/PKB, c-Kit, FLT3, and FGFR, and was evaluated in phase II clinical trials for AML patients {Tenzer, 2001 #365} {Schmidt-Arras, 2004 #377} {Chen, 2005 #354} {Stone, 2005 #337}. As we show here, the drug can influence the phosphorylation status of PAX3/FKHR and hence its transcriptional activity. Furthermore, we demonstrate a potent antitumorigenic potential of PKC412 for aRMS *in vitro* and *in vivo*. These studies suggest that targeting kinases controlling activity of oncogenic transcription factors by small molecule inhibitors is a promising therapeutic strategy against cancer.

Material and Methods

Cell lines and pharmacological inhibitors

Two human aRMS cell lines (Rh4, Rh30) and two eRMS cell lines (RD, Ruch-2) were used. The Rh4 cell line was obtained from the St. Jude Children's Hospital, Memphis, USA. The Rh30 and RD cell lines were purchased from ATCC (Rockville, MD, USA). The RUCH-2 cell line was established in our laboratories {Scholl, 2000 #368}. For ectopic expression studies of PAX3/FKHR, the human embryonal kidney cell line 293T was used (ATCC). All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % FCS (Invitrogen, Basel, Switzerland) at 37°C, 5 % CO_2.

The following compounds were used: PKI166 (EGFR/ErbB2 inhibitor), CGP59326 (EGFR inhibitor), NVP-ABG424-NX-4 (c-met inhibitor), PKC412 (N-benzoyl-staurosporine; inhibitor of different kinases, among them PKC, FGFR2/4, Flt3) and rapamycin (mTOR inhibitor). Rapamycin was purchased from Sigma (Buchs, Switzerland), all other inhibitors were provided by Novartis (Basel, Switzerland). Concentrations used in this study were chosen on the basis of preliminary dose-response experiments.

MTT Assay:

24 hours after seeding in 96-well plates (10'000 cells / well) cells were treated with pharmacological inhibitors in a volume of 100 µl medium including 10% serum for 96 hours. MTT assays (Roche, Rotkreuz, Switzerland) were then performed according to the manufacturer's protocol to measure cell numbers.

For IC_{50} determination, cells were exposed to PKC412 concentrations ranging from 0 - 10 µM. After 96 hours, cell viability was detected by MTT. IC_{50} values were calculated by nonlinear regression analysis using the GraphPad Prism software (GraphPad Software Inc., USA).

Trypan Blue Exclusion assay:

For determining cell viability, 10^6 cells were seeded in a 10 cm dish. 24 hours later, the pharmacological inhibitors were added to the medium. After 96 hours incubation, floating and adherent cells were pooled, spun down and resuspended in a defined volume of medium. After diluting the cells 1:1 with 0.4 % of trypan blue solution (Sigma, Buchs, Switzerland), alive and dead cells were counted in a Neubauer chamber.

Caspase-3 assay:

To evaluate induction of apoptosis, active caspase-3 was detected by using the CaspGLOW red active caspase-3 staining kit (Biovision, USA) according to the instructions given by the manufacturer. Briefly, 3 x 10^5 cells per 6-well were treated with 0.5 µM PKC412 for 24 - 72 hours. At each time point, floating and trypsinized cells were pooled, spun down, and incubated with Red-DEVD-FMK caspase-3 inhibitor for 45 min at 37°C. Cells positive for active caspase-3 were scored by fluorescence microscopy.

Immunoblotting:

Cells were washed once with icecold PBS and harvested by scraping in lysis buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % NP-40, 10 % glycerol) containing 1x complete protease inhibitor cocktail (Roche, Rotkreuz, Switzerland). After incubation for 15 min on ice, lysates were centrifuged at 13'000 g for 5 min and supernatants were frozen at -80°C until use. Lysates (30 µg/lane) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Protean; Schleicher & Schuell, Kassel, Germany). Membranes, blocked with 5 % BSA in TBS and Tween-20 (0.1 % w/v), were then probed with primary and HRP-coupled secondary antibodies using standard protocols. Primary antibodies used were rabbit polyclonal anti-PARP (1:XXX; Cell Signaling Technology, Danvers, USA) and mouse anti-tetra His (1:1000; Qiagen). Visualization of the antibodies was performed by using the enhanced ECL system

(Amersham, Buckinghamshire, UK).

Immunofluorescence

Cells plated on gelatine-coated glass coverslips were washed once with DPBS, fixed with 3% paraformaldehyd in PBS for 15 min, washed with DPBS and incubated with 0.1 M glycine in PBS for 5 minutes. After washing, the cells were permeabilized with 0.2% Triton X-100 in PBS for 15 minutes and washed with DPBS. The cells were then incubated with the primary antibody (anti-tetra-His, 1:200; Qiagen) in PBS containing 3% BSA for 1 hour at room temperature. After washing three times for 5 minutes with DPBS the cells were incubated with a 1:200 dilution of Alexa488-labeled secondary antibody (1:200; XXX) in PBS containing 3% BSA for 1 hour at room temperature. After washing three times for 5 minutes with DPBS, the coverslips were drained and mounted in XXX solution (). The coverslips were sealed with nail polish and examined by fluorescence microscopy.

Purification of the PAX3 DNA-binding domain protein

The PAX3 part represented in the translocation product PAX3/FKHR (AA1-391 of PAX3), including both the PAX3 paired- and homeodomain, was HIS-tagged (PAX3His) in pcDNA3 vector and transiently expressed in 293T cells. 48 hours after transfection the cells were lysed for 30 min on ice in a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 1% Triton X-100, 300 mM NaCl, 10 mM imidazole, 40 mM NaF, 10 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1mM EGTA, and 1x complete protease inhibitor mix including 1 mM EDTA (Roche, Rotkreuz, Switzerland). After centrifugation of the extract at 10'000 g for 10 min, the supernatant was incubated with 8 µl/ml 50% Ni-NTA agarose slurry (Qiagen) for 2 hours at 4°C under constant rotation. The Ni-NTA agarose was then washed 4 times with a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, and 20 mM imidazole, and 2 times with a similar washing buffer containing 50 mM imidazole. The protein was then eluted with a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl and 250 mM imidazole.

Protein purity and concentrations were determined on a bioanalyzer using protein 200 assay chips (Agilent Technologies, Waldbronn, Germany)

Desalting of the purified PAX3 protein

200 µl of the purified PAX3 solution was desalted on a C8 reversed-phase HPLC column (Brownlee Aquapore RP-300, 2.1 x 100 mm, PerkinElmer, Shelton, USA). The protein was eluted with the following HPLC program: (i) isocratic flow 0 % solvent B (v/v) for 10 min; (ii) linear gradient from 0 to 75 % B in 13 min; (iii) linear gradient from 75 to 100 % B in 5 min; and (iv) isocratic flow 100 % B for 10 min. Solvents A and B were 0.1 % trifluoroacetic acid (TFA) and 0.07 % TFA in 80 % acetonitrile, respectively.

2D-SDS-PAGE

100 µl of the desalted protein solution was dried in a vacuum centrifuge. Isoelectric focusing was performed on an IPGphor electrophoresis unit(Amersham Pharmacia Biotech, Uppsala, Sweden)

according to standard protocols (Gorg et al., 2000). Immobiline IPG strips with a non linear pH gradient of 3-10 (Amersham) were used and the samples were applied on the strips by in-gel rehydration. SDS-PAGE was carried out on a Protean II xi system (Bio-Rad, Hercules, CA, USA). Gels consisted of 13 % w/v acrylamide with 2.7 % piperazine diacrylamide (PDA) as crosslinker. Gel dimensions were 20 x 16 x 0.15 cm and running conditions were 40 mA/gel at 15 °C. Proteins were visualized by a long silver nitrate staining (Rabilloud, 2000).

Enzymatic digestion and LC-MALDI-TOF-MS analysis of the peptides

200 µl of the desalted protein solution was dried in a vacuum centrifuge. The protein was redissolved in 10 µl of a solution containing 150 mM Tris, 6 M urea, and 6 mM EDTA (pH = 8), and incubated for 2 h at 37°C. 5 µl of a solution containing 0.5 µg of endoproteinase Lys C (Roche Diagnostics, Mannheim, Germany) and 45 µl H_2O were added and incubated overnight at 37°C.

6 µl of the peptide solution was loaded on a reversed-phase capillary HPLC column (PepMap C18, 0.3 x 150 mm, LC Packings, Amsterdam, Netherlands). The peptides were separated with the following HPLC programm: (i) isocratic flow 5 % solvent B (v/v) for 5 min; (ii) linear gradient from 5 to 20 % B in 5 min; (iii) linear gradient from 20 to 60 % B in 20 min; (iv) linear gradient from 50 to 100 % B in 5 min; (v) isocratic flow 100 % B for 10 min. Solvents A and B were 0.1 % TFA and 0.07 % TFA in 80 % acetonitrile, respectively. The flow rate was 4 µl /min. Peptides eluting from the HPLC column were collected onto a 600 µm AnchorChip™ target (Bruker Daltonics, Leipzig, Germany), using a Probot microfraction collector (LC Packings), and fractions were collected in 15 s intervals. 1.5 µl of matrix solution was added to each fraction. A 1:10 dilution of saturated HCCA (α-cyano-4-hydroxycinnamic acid in 33 % CH_3CN, 0.1 % TFA) in Ethanol/Aceton 2:1 was used as matrix solution. Mass mapping was performed with an autoflex® MALDI-TOF-MS (Bruker Daltonik). Phosphorylated peptides were analyzed in the positive-ion mode with delayed extraction (60 ns) and the following voltages were applied; source 20 kV, extraction 18.80 kV, lens 7.85 kV.

In vivo labeling with [^{32}P]orthophosphate

293T cells grown in 10 cm petridishes were transfected with PAX3His or empty vector. 36 hours after transfection, cells were washed two times with phosphate-free MEM, and then incubated for 6 hours in phosphate-free MEM supplemented with 80 µCi/ml [^{32}P]orthophosphate (Phosphorous-32; GE Healthcare, Amersham, Buckinghamshire, UK). Thereafter, PAX3His was purified with the help of Ni-NTA agarose as described above. Purified proteins were then separated by SDS-PAGE and blotted onto nitrocellulose membrane, followed by detection of ^{32}P with a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

Transactivation assay

pcDNA3-PAX3/FKHR together with a reporter plasmid containing the luciferase gene downstream of PAX3 paired- or homeodomain DNA-binding sites (6xCD19 DNA-binding sites {Czerny, 1993 #346} or P3

binding site {Xia, 2004 #235}) and a plasmid containing the lacZ gene was transfected into 293T cells or electroporated into Rh4 cells using the AMAXA system (Program O17, buffer R) in ratios ensuring measurement in the linear range of the assay. Similarly, pcDNA3-FKHR together with a reporter plasmid containing the luciferase gene downstream of the bim promoter {Motta, 2004 #217} and a plasmid containing the lacZ gene was transfected into 293T cells in a ratio of 1:1:0.01.
16 hours later the cells were incubated with PKC412 or DMSO vehicle for 24h, followed by lysis in reporter lysis buffer (Promega, Madison, WI, USA). ß-Galactosidase and luciferase activities were determined with the corresponding assay systems (Promega). Luciferase activity values were normalized with the ß-galactosidase activity values.

Mutagenesis

PAX3/FKHR mutants were generated using the "GeneTailor site-directed mutagenesis system" (Invitrogen) according to the manufactorer´s instructions. All constructs were sequenced for verification.

Silencing of PAX3/FKHR

SiRNA mediated silencing of PAX3/FKHR was performed as described previously (Ebauer et al.).

Real time PCR

1 µg RNA was used for cDNA synthesis with oligo-dT primers using the Omniscript kit (Qiagen) including DNAse treatment. Then, 5 to 10 ng cDNA was used as template for the PCR reaction under universal cycling parameters on an ABI7900 instrument using commercially available target probes ("Assay-On-Demand") for the indicated genes and Mastermix (all from Applied Biosystems, Applera Europe BV, Rotkreuz, Switzerland). Detection of PAX3/FKHR was achieved using PAX3 forward (5'GCACTGTACACCAAAGCACG3') and FKHR reverse (5'AACTGTGATCCAGGGCTGTC3') primers applying the fluorescent SYBR green method (Applied Biosystems). Cycle threshold (C_T) values were normalized to GAPDH. Relative expression levels of the target genes among the different samples were calculated using the $\Delta\Delta C_T$ method.

RMS xenograft studies:

10^7 Rh4 or Rh30 cells in 100 µl of PBS were injected into the right flank of athymic CD1 nude (nu/nu) mice (Charles River, Germany). When tumors reached a volume of 130 - 200 mm^3, oral treatment with PKC412 (100 mg/kg/day) or placebo for 15 days was started. Tumor diameters (d_1, d_2) were measured 2 -3 times a week using a digital calliper. Tumor volumes were calculated by using the following formula: $V = (4/3)\ \pi\ r^3$, where $r = ((d_1 + d_2)/4)$. At the end of the treatment period, mice were sacrificed, tumors were isolated and fixed in 4 % paraformaldehyde in PBS. Paraffin-embedded tumor sections were subjected to hematoxylin and eosin (H&E) stainings. Cell proliferation was assessed by Ki-67 stainings using a monoclonal rabbit Ki-67 antibody (1:XXX; clone SP6; NeoMarkers, Fremont, USA) and a biotinylated anti-rabbit IgG antibody (1:XXX; Jackson ImmunoResearch, West Grove, USA), respectively. Immunostaining was performed

using a standardized automated procedure on a Ventana Benchmark system (Ventana Medical Systems, Tucson, USA). Furthermore, apoptosis was detected by terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling (TUNEL) according to the instructions of the manufacturer (in situ cell death detection kit, Fluorescin, Roche, Rotkreuz, Switzerland). Ki-67 or TUNEL positive cells were scored in 5 - 10 randomly selected visual fields at x200 (Ki-67) or x160 (TUNEL) magnification.

Results

Influence of kinase inhibitors on growth of RMS cells

Recent gene expression studies of RMS biopsies {Davicioni, 2006 #335} {Wachtel, 2004 #257} identified a series of receptor tyrosine kinases (RTKs) with potential oncogenic capabilities highly expressed in embryonal and/or alveolar RMS. Among these are EGFR (ErbB1), which is highly expressed in eRMS, and HGFR (c-met) and FGFR2/4, which show high expression levels in aRMS. Therefore, as these RTKs represent interesting putative targets for RMS treatment, we tested a series of small molecule inhibitors targeting them in comparison to rapamycin as mTOR inhibitor {Hosoi, 1999 #332} for potential growth inhibitory effects on two aRMS (Rh4, Rh30) and two eRMS (RD, Ruch-2) cell lines. Expression of the relevant target genes in these cell lines was verified in gene expression data acquired from these cells and corresponded to the expected pattern (data not shown). The experiments were carried out according to the same general outline. Inhibitors were tested for antiproliferative effects in dose response experiments as assessed by MTT assay (Fig.1A). Then, induction of cell death by the different inhibitors was measured by trypan blue exclusion assays (Fig.1B). For the individual drugs, we made the following observations:

Rapamycin, the inhibitor of mTOR, is known to induce apoptosis in Rh30 cells when cultured in serum-free medium. In the presence of serum, rapamycin induces cell cycle arrest {Hosoi, 1999 #332}. In our experiments, which were all carried out under serum-containing conditions, we could confirm the growth inhibitory effects of rapamycin in RMS. Both, aRMS and eRMS cells showed a reduced metabolic activity in the MTT assays (Fig.1A). However, no significant loss of cell viability was observed (Fig.1B).

PKI166 and CGP59326 are known to block the activity of either both EGFR (ErbB1) and ErbB2, or only EGFR, respectively. In RMS cell lines, CGP59326 affected neither cell growth nor viability (Fig.1A,B). IN contrast, PKI166 inhibits growth of aRMS cells, more efficiently than eRMS (Fig.1A), but without significant induction of cell death (Fig. 1B), indicating rather inhibition of proliferation than induction of cell death in aRMS cell lines.

The c-met inhibitor NVP-ABG424-NX-4 showed in all RMS cell lines only a moderate influence on cell growth except for RD cells (Fig. 1A). RD cells also exhibited a dramatic induction of cell death, suggesting that they are the only RMS cells tested which are sensitive to this drug.

Finally, PKC412 which is known to inhibit FGFR (but also various other kinases such as PKC, PKB/Akt, Flt-3 and c-kit), inhibited proliferation of Rh4 and Rh30 much more effective than that of RD cells (Fig. 1A).

Also RUCH-2 cells showed a reduced proliferative activity. However, cell death is significantly induced only in the two aRMS and not in eRMS cells (Fig. 1B).

Taken together, among the inhibitors tested, PKC412 demonstrates the most efficient anti-proliferative and cell death inducing effect specifically for aRMS cells. The current lack of alternative drugs for treatment of metastasized aRMS makes this finding especially interesting. Therefore, PKC412 was further characterized.

PKC412 induces apoptosis in aRMS cells

Therapeutically significant IC_{50} levels for PKC412 were only achieved in aRMS, but not in eRMS cells (Fig.2A). To investigate whether cell death is due to apoptosis, we used an activated caspase-3 assay. As shown in Fig. 2B, induction of caspase-3-dependent apoptosis dramatically increases in aRMS cells when treated with 0.5 μM PKC412 for 24-72 hours. In contrast, apoptosis is only slightly induced in eRMS cells. This finding was confirmed by examining cleavage of the caspase-3 substrate PARP (Fig.2C), indicative for active caspase-3 and therefore ongoing apoptosis, as early as 4 hours after start of incubation with 0.5 μM PKC (Fig. 2B). Therefore, PKC412 selectively induces caspase-3 dependent apoptosis in alveolar but not in embryonal RMS cells.

PKC412 influences the transcriptional activity of PAX3/FKHR

As first demonstrated a decade ago, aRMS cells undergo apoptosis upon silencing of PAX3/FKHR by oligonucleotides {Bernasconi, 1996 #333} {Margue, 2000 #64} or siRNA (manuscript submitted), demonstrating that aRMS cells are strongly addicted to active PAX3/FKHR.

Hence, we argued that PKC412 might have an influence on PAX3/FKHR activity. In the absence of PKC412 ectopic expression of PAX3/FKHR in HEK 293T cells induces transcription of the PAX3/FKHR-target gene CB1 from undetectable levels in a dose dependent manner, as detected by qRT-PCR (Fig.3A). A point mutation (N269A) within the homeodomain of PAX3, specifically inactivating its homeodomain-DNA binding {Xia, 2004 #235}, prevents this induction of CB1 transcription (Fig.3A), but not of the paired domain dependent target gene AP2β (data not shown). This strongly suggests that induction of CB1 transcription in 293T cells is a direct effect of PAX3/FKHR on the promoter of this gene and not indirect via other proteins, confirming the results of other studies {Begum, 2005 #256}. Furthermore, also in Rh4 cells, the bulk part of the CB1 expression measured is PAX3/FKHR dependent, as specific silencing of PAX3/FKHR by siRNA reduced CB1 expression to less than 20 percent compared to scrambled control (Fig.3B). The transcription level of this gene was therefore used as an indicator for PAX3/FKHR activity in further experiments. In presence of PKC412, PAX3/FKHR-induced transcription of CB1 in 293T cells is inhibited in a dose-dependent manner, whereas expression of housekeeping genes used for normalization such as GAPDH was uninfluenced (Fig.3C). Similarly, endogenous mRNA levels of CB1 were reduced in Rh4 cells upon PKC412 treatment (Fig.3C). This inhibitory effect of PKC412 was further validated by conventional transactivation assays using luciferase reporter plasmids containing specific promoters for the paired domain (6xCD19) or the homeodomain (P3) of PAX3. Again in these

assays PKC412 strongly reduces PAX3/FKHR induced expression of the reporter in both 293T cells and Rh4 cells (Fig.3D).

To exclude that PKC412 mediates these effects via downregulation of PAX3/FKHR expression, mRNA as well as protein levels of PAX3/FKHR were measured. Interestingly and contrary, incubation of PAX3/FKHR-transfected 293T cells with PKC412 led to an increase of PAX3/FKHR on the mRNA level as measured by qRT-PCR (Fig.3E) as well as on the protein level as determined by Western blot analysis (Fig.3F). Taken together, these results suggest that PKC412 modulates PAX3/FKHR activity and that these effects are rather under- than overestimated.

To evaluate whether the PAX3- or the FKHR-part of the fusion protein are targets of the PKC412 mediated effect, we measured the influence of PKC412 on the transactivation activity of the alternative translocation product PAX3/NCOA1 {Wachtel, 2004 #257} and on FKHR alone. This approach demonstrated that PKC412 inhibits transcriptional induction of CB1 by PAX3/NCOA1 very similar to PAX3/FKHR (Fig.3G). In contrast, the transactivation potency of FKHR on a luciferase reporter plasmid driven by the promoter of the FKHR-target gene bim {Motta, 2004 #363} was unchanged or slightly stimulated by PKC412 in a dose dependent manner (Fig.3H). Taken together, these data suggest that it is rather the PAX3 part than the FKHR domain of the fusion protein that is influenced by PKC412.

The PAX3 part of PAX3/FKHR is phosphorylated at multiple sites

As broad spectrum kinase inhibitor, we hypothesized that PKC412 might affect phosphorylation(s) status as a mechanism to reduce the activity of PAX3/FKHR. Up to date, several phosphorylation sites targeted by PKB/Akt, p38 and Erk have been described in the FKHR part of the fusion protein {Rena, 1999 #334; Rena, 2002 #21; Brunet, 1999 #234; Asada, 2006 #364}. However, at least the sites targeted by PKB/Akt, which is known to be inhibited by PKC412 {Tenzer, 2001 #365}, mainly regulate FKHR subcellular localization and are thought to have no function in the context of the permanently nuclear localized PAX3/FKHR fusion protein {Del Peso, 1999 #20}. Therefore, and since PAX3/FKHR and PAX3/NCOA1 behave similarly, we examined the phosphorylation state of the PAX3 part of PAX3/FKHR directly using a His-tagged form (PAX3His). Immunohistochemistry of transfected 393T cells with an anti-His-tag antibody proved that PAX3His is localized in the nucleus of the cells, as known for full lenght PAX3/FKHR (Fig.4A). Furthermore, incubation with 10 μM PKC412 for 24h did not change the localization of the protein. PAX3His was then purified from ^{32}P-labeled 293T cells and tested for potential phosphorylation. As demonstrated in Fig.4B, a strong ^{32}P-signal was detected in the PAX3His protein, directly suggesting that PAX3 is a phosphoprotein. Analysis with 2D-gel electrophoresis further supported this conclusion, as the purified PAX3-His protein revealed to be a mixture of several species with different pIs, reminiscent of multiple phosphorylation sites in a protein (Fig.4C). Finally, LC-MALDI-TOF mass spectrometric analysis of LysC-digested PAX3-His protein directly revealed 4-5 phosphates in the peptide 186-216 (m/z of 4-fold phosphorylated peptide is 3602, m/z of 5-fold phosphorylated peptide is 3683) (Fig.4D). This peptide includes the so called octapeptide of PAX3 and contains six Ser residues (S187, S193, S197, S201, S205, S209) as potential phosphorylation sites (Fig. 4E). Four out of these six Ser (S187, S201, S205,

S209) are conserved among the PAX3 proteins from different species suggesting irreplaceablity for proper function of PAX3 (Fig.4F). Interestingly, *in silico* analysis of the probability for phosphorylation using the DISPHOS1.3 web resource (http://core.ist.temple.edu/pred/), which uses disorder information to predict phosphorylation, revealed that these six Ser residues and the two flanking S180 and S222 have the highest probability for being phosphorylated among all potential phosphorylation sites in PAX3 (data not shown).

Phosphorylation influences activity of PAX3/FKHR

To test whether PKC412 influences the phosphorylation status of PAX3His, protein purified from PKC412- and control-treated 293T cells was compared by 2D-gel electrophoresis. As shown in Fig. 5A, PKC412 induces a clear shift of the PAX3His species towards more basic pIs. Furthermore, electrospray ionization (Fig.5B) as well as MALDI-TOF (data not shown) mass spectrometric analysis of the same proteins revealed a PKC412-treatment induced shift towards smaller protein species. These results suggest that PKC412 affects the post-translational modification of PAX3.

To test whether these phosphorylation sites are directly affecting PAX3/FKHR activity, we mutated the six Ser in the phosphorylated peptide individually into Asp, to mimic phosphorylation and potentially prevent PKC412 mediated inhibition. However, these single mutations did not affect sensitivity of PAX3/FKHR towards PKC412 (data not shown). Instead, mutation of all six Ser altogether into Asp partially rescues transcriptional activity of PAX3/FKHR (Fig.5C), suggesting involvement of more than one of these sites in regulation of PAX3/FKHR activity. Additional mutation of S180 into Asp did not lead to a significant further increase in rescue (Fig.5C), whereas mutation of S222 led to complete loss of transactivation activity (data not shown). Therefore, activating mutations can be inhibited by PKC412 to a lesser extant than the wild type protein. Furthermore, loss-of-function mutation of the six Ser into Ala led to a loss of transactivation activity as measured in transactivation assays using the 6xCD19 reporter plasmid by about 50% (Fig.5D). We conclude that indeed phosphorylation plays a role in regulation of PAX3/FKHR activity and can be influenced by PKC412 treatment.

Inhibition of *in vivo* tumor growth of aRMS xenografts by PKC412

Kinase inhibitors are interesting molecules for potential treatment of aRMS, especially when they are able to affect activities of key oncogenes such as PAX3/FKHR. Therefore, we further investigated the effects of PKC412 on tumor growth *in vivo*. Towards this end, Rh4 and Rh30 xenograft mice were treated daily by oral administration of PKC412 (100 mg kg^{-1}). Treatment was maintained for 15 days and tumor size measured every 2 - 3 days. In both xenograft models tumor growth was significantly inhibited by PKC412 treatment (Fig. 6A). In Rh4 xenografts a complete suppression of tumor growth was observed, whereas in Rh30 xenografts tumor growth was strongly decreased by PKC412.

To further characterize the effect of PKC412 on xenograft tumors, mice were sacrificed at the end of the treatment period, tumors isolated, and stained by immunohistochemistry. Tumor morphology was determined by H&E stainings. Placebo-treated Rh4 (Fig. 6B, upper line) and Rh30 (Fig. 6C, upper line)

xenograft sections both showed a high density of actively growing tumor cells. In contrast, PKC412 treated tumors (Fig. 6B, lower line) showed a dramatic increase in connective tissue.

In order to quantify the influence of PKC412 on tumor cell proliferation *in vivo*, tumor sections were immunohistochemically stained for the proliferation marker Ki-67 and apoptotic cells were detected by TUNEL staining. As shown in Fig. 6B, the number of Ki-67 positive cells is decreased in both Rh4 and Rh30 xenografts after treatment, whereas the number of apoptotic tumor cells dramatically increased by 4 - 5 fold in tumors isolated from both Rh4 and Rh30 xenograft mice treated with PKC412.

In summary, PKC412 significantly inhibited tumor growth, reduced numbers of actively dividing tumor cells while simultaneously increasing number of apoptotic cells.

Discussion

Aberrant constitutive activation of signaling cascades involved in regulation of differentiation, cell division and apoptosis is characteristic for many tumors and is thought to be a fundamental cause for tumorigenesis, promoting both tumor development and progression. Importantly, in many tumor types, tumors cells have been shown to acquire a dependency on such constitutive signaling and are growth inhibited or undergo apoptosis upon inhibition of these cascades, a phenomenon named oncogene addiction {Weinstein, 2002 #361; Weinstein, 2006 #362}. Therefore, components of these aberrant signaling pathways represent potential ´Achilles` heels` of the tumors and are postulated to be promising targets for tumor treatment. Especially upstream components of these cascades such as growth factor receptor kinases or cytoplasmic signal transduction kinases have been proposed as targets due to their good accessability for inhibitory antibodies or drugable enzymatic activities. In some cases the use of inhibitors against particular aberrant kinases has already made the way to the clinics as supplemental or even monotherapeutic alternative approach to conventional tumor therapies. Up to date, eight kinase-targeted oncology drugs have received regulatory approval (for a review see {Sebolt-Leopold, 2006 #348}), most prominently in case of the bcr-abl kinase inhibitor Gleevec (Imatinib) for CML treatment {Cohen, 2002 #378} or the EGFR inhibitors Gefitinib (Iressa) {Cohen, 2004 #379}, and Erlotinib (Tarceva) {Johnson, 2005 #380} for treatment of non small cell lung cancer. More than 100 additional agents are currently undergoing clinical evaluation.

In case of RMS, the current treatment is still based on conventional treatment regimens (surgery, chemotherapy and radiotherapy), which in the case of metastasized aRMS often fail. Alternative treatment agents are therefore highly desired.

In an approach to find targetable pathways in RMS, we therefore analyzed our recent gene expression data of RMS biopsies {Wachtel, 2004 #257} for the expression of kinases with a known tumor-relation and tested a series of kinase inhibitors against three selected kinases (EGFR, HGFR, FGFR), in comparison with mTOR which has been characterized as target in RMS *in vitro* {Hosoi, 1999 #347}, for their ability to reduce the growth of different aRMS and eRMS cell lines.

From all substances tested, PKC412 (N-benzoylstaurosporine; midostaurin; CGP41251) was found to have the most promising anti-growth effect. This compound induces very efficiently apoptosis in aRMS cell lines and also in aRMS xenografts in nude mice in submicromolar concentrations. This is well below the steady-state plasma levels of 2-7 μM, which were achieved in phase I clinical trials with this substance {Propper, 2001 #357}. As these concentrations were well tolerated by patients, our *in vitro* data provide a potential preclinical rationale for clinical studies of aRMS treatment.

PKC412 is a derivative of staurosporine and was developed as PKC inhibitor. Although being less unspecific when compared to staurosporine, which is known to bind to a bulk of the whole kinome {Fabian, 2005 #271}, also PKC412 was subsequently found to inhibit a wide range of other kinases such as cdk1/cycB, PKA, c-src, KDR (VEGFR2) and others in submicromolar concentrations {Fabbro, 1999 #336}. Based on these characteristics, PKC412 has been successfully applyed as inhibitor of different oncogenic kinases in a range of tumors *in vitro*, e.g. as FGFR inhibitor in myeloproliferative disorder or multiple myeloma {Chen, 2004 #355; Chen, 2005 #354}, as kit inhibitor in mast cell leukemia {Gotlib, 2005 #356; Gleixner, 2006 #359} or as Akt inhibitor in myeloma {Bahlis, 2005 #360}. Its potential clinical use is most advanced as Flt3 inhibitor for the treatment of AML, for which PKC412 is used in a phase II clinical study {Stone, 2005 #337}.

In this study, an inhibitory effect of PKC412 on transcriptional activity of PAX3/FKHR, an important potential therapeutic target in RMS, was detected. This effect of PKC412 is reflected e.g. by its ability to inhibit the induction of target gene transcription upon ectopic expression in the non-RMS cell line 293T. In the aRMS cell line Rh4 the endogenous transcription of these target genes is also affected by PKC412, albeit less pronounced. However, while 293T cells do well tolerate even 10 μM PKC412, aRMS cells rapidly undergo apoptosis upon PKC412 treatment, potentially interfering with determination of target gene levels. In contrast, when using reporter-plasmid based transactivation systems to measure PAX3/FKHR activity, the effects of PKC412 in 293T and Rh4 cells were comparable, suggesting effective inhibition of PAX3/FKHR activity by PKC412 also in Rh4 cells.

We found that the inhibitory effect of PKC412 on PAX3/FKHR activity is at least partially based on modulation of phosphorylation sites in the PAX3 part of the fusion protein. While phosphorylation of PAX2 {Cai, 2002 #341}, PAX6 {Mikkola, 1999 #343} {Kim, 2006 #342} and PAX8 {Poleev, 1997 #338} {Van Renterghem, 1996 #339} has been reported, phosphorylation sites in PAX3 have not been published so far. However, an influence of PKC on the transcriptional activity of PAX3 in presomitic mesoderm has been suggested in a recent work {Brunelli, 2007 #381}. Interestingly, while the phosphorylation sites in PAX2,6,8 have either not been elucidated or are located in the transactivation part of the protein, in the highly homologous DNA binding part of the PAX proteins no phosphorylation has been reported up to date. The region encompassing phosphorylation sites in PAX3 is located in the DNA binding part between the two DNA binding domains of PAX3 in the so called octapeptide region of PAX3. The octapeptide is a conserved region found in 7 out of 9 PAX family members, and in the case of PAX5 it has been shown to be involved in protein-protein interaction with the corepressor groucho-4 thereby influencing its

transcriptional activity {Eberhard, 2000 #301}. The fact that also PAX3 interacts with groucho-4 {Lang, 2005 #344} suggests that a similar mechanism might operate in PAX3.

MALDI-TOF analysis revealed that at least 4 out of 6 Ser residues are phosphorylated in the peptide 186-216 of PAX3. Interestingly only multiple but not single mutations of these sites into Asp did reduce the sensitivity of the protein towards PKC412, suggesting that a combination of several phosphorylated sites is necessary for full transcriptional activity of PAX3/FKHR. As multisite phosphorylation is in many cases a cooperative and coordinate event, mutation of one phosphorylation site might not reveal the true function of that site (for review see {Holmberg, 2002 #278}). Whether all or a combination of some of these sites is involved in PKC412-sensitivity is under current investigation. Furthermore, the fact that exchange of all the six Ser by Asp did not completely protect from PKC412-mediated inhibition of PAX3/FKHR suggests that beside modulation of these phosphorylation sites also other mechanisms regulating PAX3/FKHR activity are affected by PKC412.

Translocation-positive aRMS cells have been shown to highly depend on the presence of active fusion proteins, as silencing of PAX3/FKHR by oligonucleotides {Bernasconi, 1996 #333} {Margue, 2000 #64} or siRNA (manuscript submitted) very efficiently induces apoptosis in these cells. This suggests that reduction of PAX3/FKHR activity by PKC412 in aRMS cells may be, at least in part, the responsible mechanism for the detected induction of apoptosis. As tumors have many, and often overlapping, biological pathways they can use to grow and resist death, the current opinion is that inhibition of different signal transduction pathways in parallel may be more effective when compared to therapies targeting specifically one single pathway (for review see {Faivre, 2006 #350}). In this context, the diverse inhibitory effects of PKC412 could be advantageous. Furthermore, such multitarget therapy is thought to have an increased likelihood of sustained effectiveness due to the reduced probability for appearance of resistant clones.

About one third of the known cellular oncogenes are transcription factors {Karamouzis, 2002 #349} {Futreal, 2004 #307} therefore these factors are highly interesting targets for potential therapeutic interventions. Unfortunately, the absence of a directly targetable enzymatic activity complicates the targeting of these factors. Experimental approaches to inhibit transcription factors, such as antisense nucleic acid approaches have been shown to be very useful *in vitro*, also in the case of aRMS {Bernasconi, 1996 #333}, but the technical complexity of an application *in vivo* prevents routine clinical implementation for the moment. The use of small molecule inhibitors influencing transcription factor activity could be an alternative. As demonstrated in our study, they may not target transcription factors directly, but kinases that regulate their activity. This approach would have the advantage that small molecule inhibiting a well characterized class of enzymes (kinases) could be used. Furthermore, as most of the approximate 1000 human transcription factors are thought to be regulated by phosphorylation {Gardner, 2005 #280}, the principles shown here may be worthwhile to explore for additional tumors addicted to oncogenic transcription factors such as e.g. Ewing's sarcoma.

In summary, our data reveal PKC412 as an interesting potential agent for the treatment of aRMS and provide a preclinical rationale for clinical studies with this inhibitor in aRMS patients.

Acknowledgement

Expression constructs with the PAX3/FKHR mutants G48S and N269A were kindly provided by Prof. F.G.Barr. The 6xCD19 and P3 luciferase constructs were kindly provided by Dr. M. Busslinger. The bim-luciferase reporter construct was kindly provided by Dr. R.H. Medema. We thank Dr. W. Jochum for his help with Ki-67 immunohistochemical stainings of tumor sections. This study was supported by a grant from the Swiss National Science Foundation (3100-xxxx) to BWS.

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Figure Legends

Fig.1. Screening of small molecule inhibitors for inhibitory effects on growth of aRMS (Rh4, Rh30) and eRMS (RD, Ruch-2) cell lines**.** (A) Changes in proliferative activity as measured by MTT assays. Small molecule inhibitors presumably targeting EGFR/ErbB2 (PKI166), EGFR (CGP59326), c-met (NVP-ABG424-NX-4), FGFR (PKC412), and mTOR (Rapamycin) were applied for 96 hours. Each inhibitor was tested at three concentrations (indicated in µM). *Columns*, mean of three independent experiments performed in triplicate; *bars*, SD. (B) Cell death was assessed using trypan blue exclusion. RMS cells were treated with a moderate concentration of the inhibitors described in (A) for 96 hours. *Columns*, mean of three independent experiments; *bars*, SD.

Fig.2. PKC412 induces apoptosis in aRMS but not in eRMS cell lines. (A) IC_{50} values of PKC412 as determined by MTT assays of RMS cells treated with graded concentrations of PKC412 (0 - 10 µM) for 96 hours. Note: the IC_{50} value for RD cells was not determinable (n. d.) because a 50 % inhibition of proliferative activity could not be achieved by using the corresponding PKC412 concentrations. *Columns*, mean of three independent experiments performed in triplicate; *bars*, SD. (B) Evaluation of apoptotic cells

by an activated caspase-3 assay after treatment with 0.5 µM PKC412 (X) or DMSO ( ) for 24 – 72 hours. *Points*, mean of three independent experiments; *bars*, SD. (**C**) Western blot analysis of PARP cleavage performed on lysates (15 µg/lane) of RMS cells after treatment with 0.5 µM PKC412 or DMSO for 2, 4, 8 and 16 hours.

Fig.3. PKC412 inhibits the transcriptional activity of PAX3/FKHR. (A) Induction of transcription of the PAX3/FKHR target gene CB1 in 293T cells upon ectopic expression of the indicated PAX3/FKHR constructs as measured by qRT-PCR. Results of one representative experiment are shown. (B) Effect of siRNA-mediated silencing of PAX3/FKHR on the transcription level of CB1 in Rh4 cells as measured by qRT-PCR at the indicated time points. (C) Effect of PKC412 on ectopic PAX3/FKHR-induced CB1 transcription in 293T cells and on endogenous CB1 transcription in Rh4 cells as measured by qRT-PCR. Cells were incubated with the indicated concentration range of PKC412 for 16h. Mean and SD of three independent experiments are shown. (D) Effect of PKC412 on the transactivation potency of PAX3/FKHR on two luciferase reporter plasmids containing specific promoters for the paired domain (6xCD19) or the homeodomain (P3) of PAX3. Rh4 or 293T cells transfected with PAX3/FKHR together with the indicated reporter plasmid were incubated with the indicated concentration of PKC412 for 16h. Mean and SD of three independent experiments are shown. (E) Effect of PKC412 on the level of PAX3/FKHR transcript in PAX3/FKHR transfected 293T cells as measured by qRT-PCR. Cells were treated as described in B. (F) Effect of PKC412 on the level of PAX3/FKHR protein in PAX3/FKHR transfected 293T cells as measured by Western blot detection with an anti-PAX3 antibody. Cells were treated as described in B. (G) Comparison of the effects of PKC412 on the transcriptional activation of CB1 by PAX3/FKHR and PAX3/NCOA1. 293T cells were transfected with the indicated PAX3-fusion protein construct and incubated with PKC412 for 16h. (H) Effect of PKC412 on the transactivation potency of FKHR on a luciferase reporter plasmid driven by the promoter of the FKHR-target gene bim. 293T cells transfected with FKHR and the bim reporter plasmids were incubated with the indicated concentration range of PKC412 for 16h. Mean and SD of three independent experiments are shown.

Fig.4. The PAX3 part of PAX3/FKHR is phosphoylated *in vivo*. (A) Immunofluorescent detection of the His-tagged PAX3-part from PAX3/FKHR (PAX3His) with an α-tetra-His antibody in transfected 293T cells cultivated in presence or absence of 10 μM PKC412 for 24h. (B) Phosphoimager detection of ^{32}P (left panel) and α-tetra-His immunodetection (right panel) of Western blotted PAX3His protein purified from ^{32}P labeled 293T cells. (C) Silver stained 2D-gel of PAX3His protein purified from 293T cells (lower panel) and α–tetra-His immunodetection of a blotted 2D gel of the same protein (upper panel). (D) MALDI-TOF mass spectrum of the peptide aa186-216 of PAX3. PAX3His was purified from PAX3His-transfected 293T cells and digested by LysC. Resulting peptides were isolated by chromatography and investigated for phosphorylation by MALDI-TOF-MS. (E) Schematic representation of the domain structure of PAX3 depicting the localization of the identified phosphorylation sites in PAX3. (F) Alignment of the PAX3 protein sequences from the indicated species including the region with the identified phosphorylation sites.

Phosphorylation sites conserved among all four species (S187, S201, S205, S209) are indicated by arrows.

Fig.5. Partial rescue from inhibition by PKC412 by phosphate mimicking mutations in PAX3/FKHR. (A) Silver stained 2D-gel of PAX3His protein purified from transfected 293T cells incubated in absence (upper panel) or presence (lower panel) of 10 μM PKC412 for 16h. (B) ESI-MS analysis of the same proteins as in A. (C) Effect of PKC412 on induction of CB1 transcription in 293T cells upon ectopic expression of wildtype PAX3/FKHR or PAX3/FKHR with Ser-Asp exchanges of the indicated amino acids as measured by qRT-PCR. Mean and SD of three independent experiments are shown. (D) Relative transactivation potencies of wildtype PAX3/FKHR or PAX3/FKHR with the indicated Ser-Ala mutations on the 6xCD19 reporter plasmid as measured by luciferase assays. Mean and SD of three independent experiments are shown. Asterisks represent $P<0.05$ vs. wt.

Fig.6. *In vivo* anti-tumor effects of PKC412 in two different aRMS xenograft models. (A) Growth inhibition of Rh4 and Rh30 xenografts in female CD-1 athymic nude mice (nu/nu) treated with a daily dose of 100 mg/kg PKC412 for up to 15 days. (B) Evaluation of proliferation and apoptosis in PKC412-treated xenografts by immunohistochemistry and TUNEL staining. After treatment, tumors were excised, fixed and paraffin-embedded. Sections were stained for Ki-67 (*upper row)* or TUNEL-stained (*lower row*). Proliferation and apoptosis were quantified by counting Ki-67- and TUNEL-positive cells. *Columns*, means for each treatment group (n = 3 - 6); *bars*, SD. (**C**) Haematoxylin and eosin (H&E) stainings of Rh4 (A - D) and Rh30 (E - H) xenograft sections. *Upper row*, placebo-treated tumors; *lower row*, PKC412-treated tumors. Cells within the square in left panels are shown in right panels at higher magnification (Bar 200 μm (*left panels*) and 100 μm (*right panels*).

Figure S1. Effects of PKC412 on phosphorylation of signal transduction proteins. Western blot analysis of p-AKT, p-FGFR1-4, p-4E-BP1, p-p70/85 S6 kinase, p-p44/42 and p-IGFR-I were performed in cell lysates (30 μg/lane) from Rh4 cells after treatment with indicated concentrations of PKC412 for 10 minutes. Blots were stripped and reprobed with anti-ß-catenin as a loading control.

Fig. 1 A

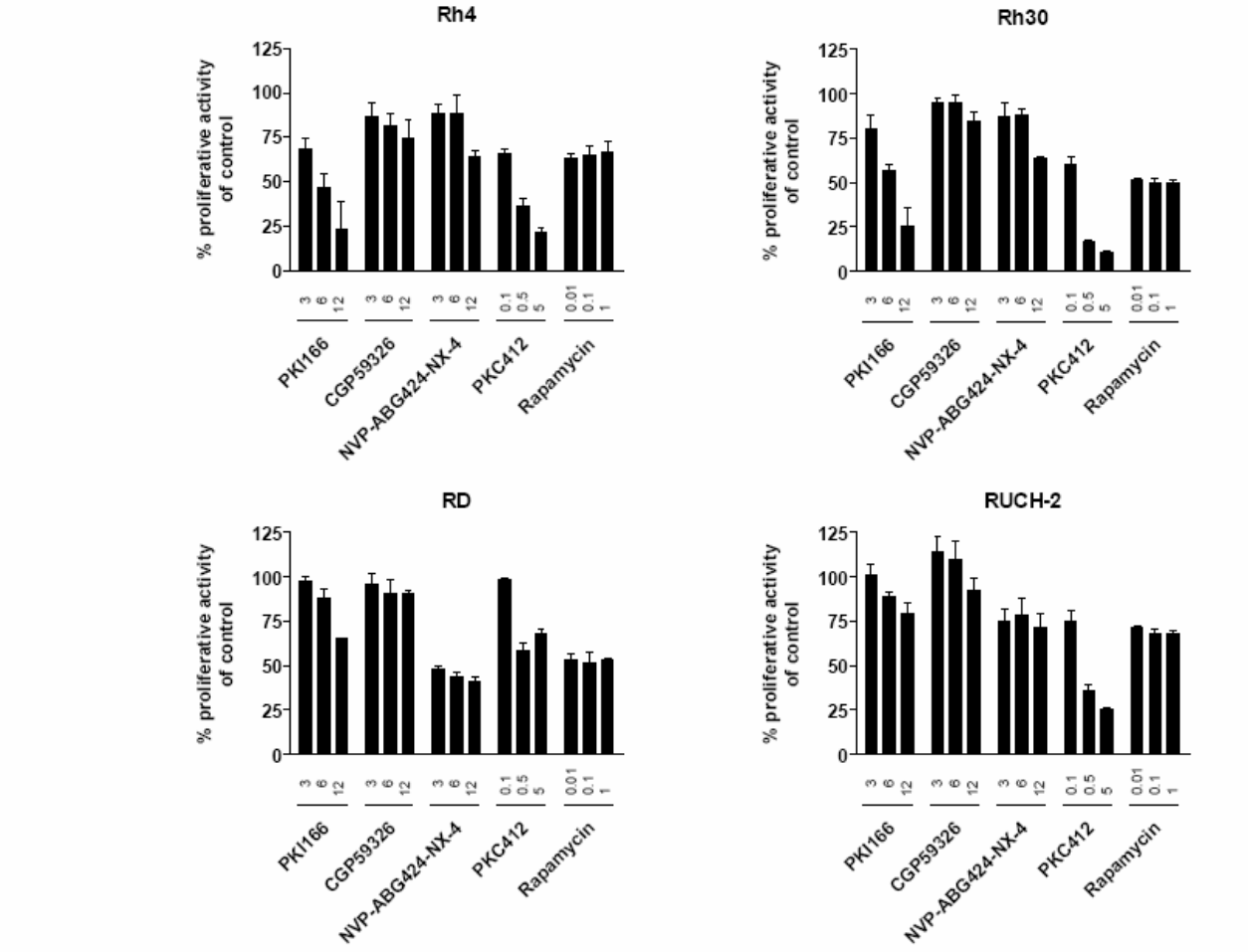

Rh4
Rh30
RD
RUCH-2
% proliferative activity of control
125
100
75
50
25
0
3
6
12
0.1
0.5
5
0.01
0.1
1
PKI166
CGP59326
NVP-ABG424-NX-4
PKC412
Rapamycin

Fig. 1 B

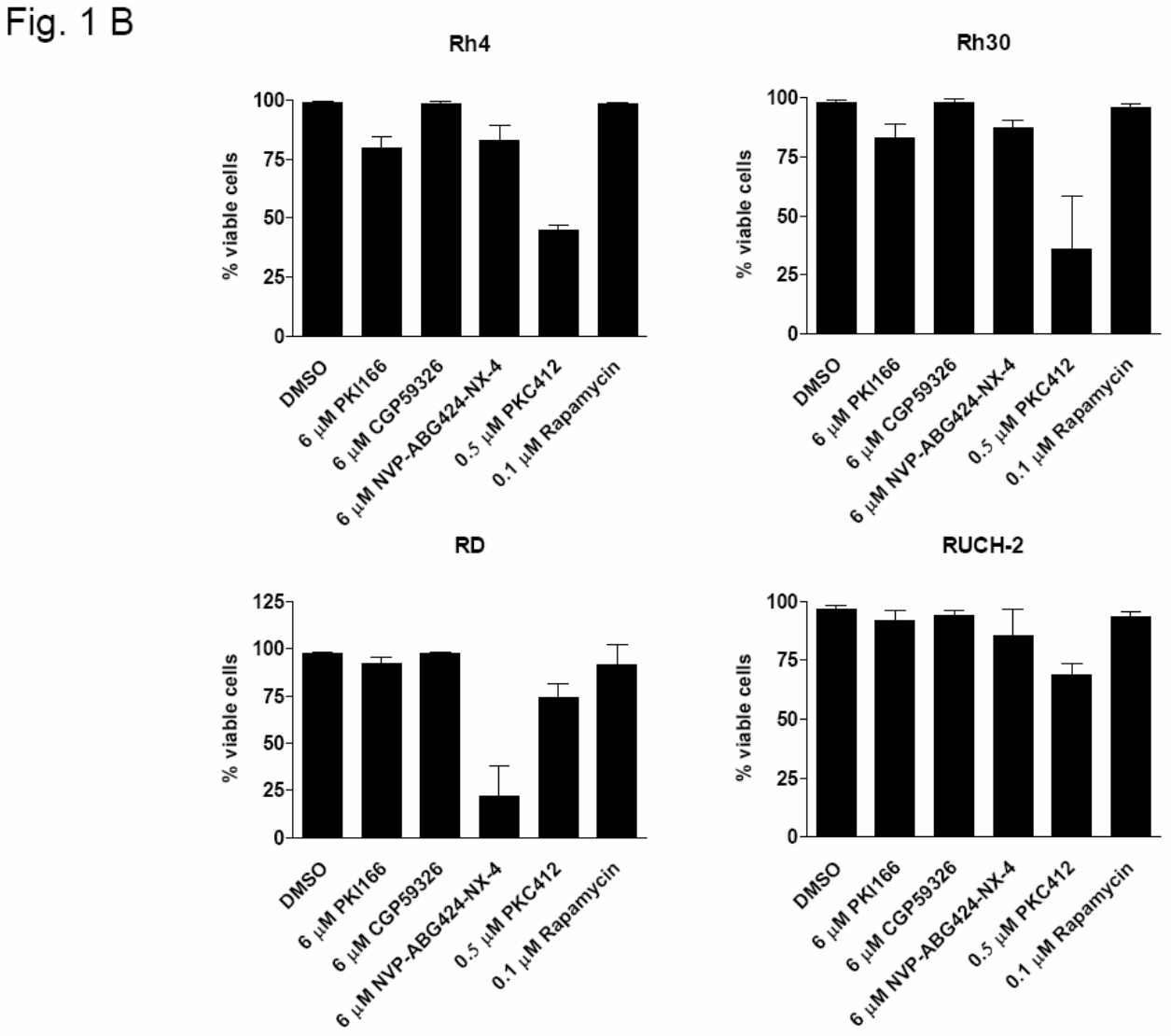
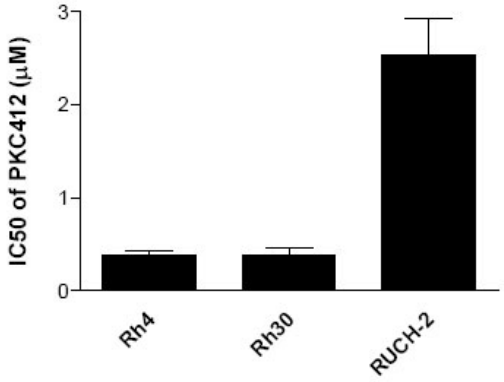

Rh4
Rh30
RD
RUCH-2
% viable cells
0
25
50
75
100
125
DMSO
6 μM PKI166
6 μM CGP59326
6 μM NVP-ABG424-NX-4
0.5 μM PKC412
0.1 μM Rapamycin

Fig. 2A:



	IC50 (µM)
Rh4	0.38 ± 0.05
Rh30	0.39 ± 0.07
RD	n. d.
RUCH-2	2.53 ± 0.40

Fig. 2B:

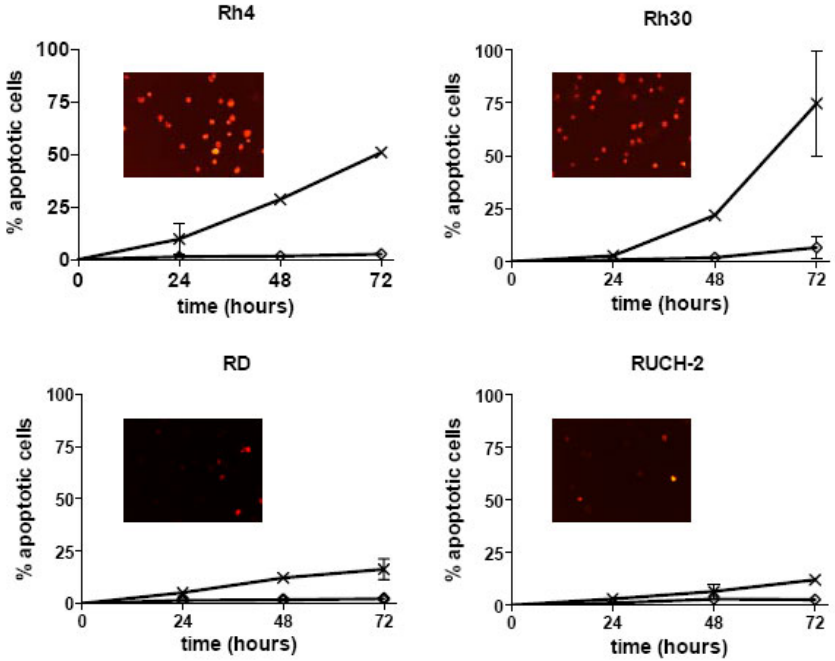
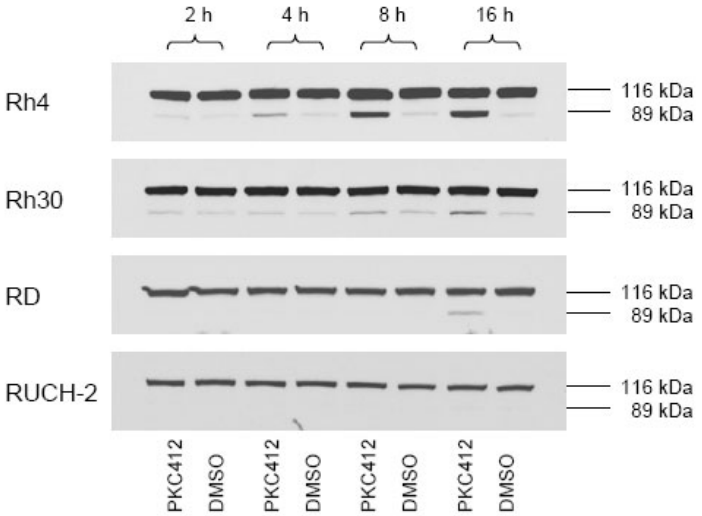


Fig. 2C:



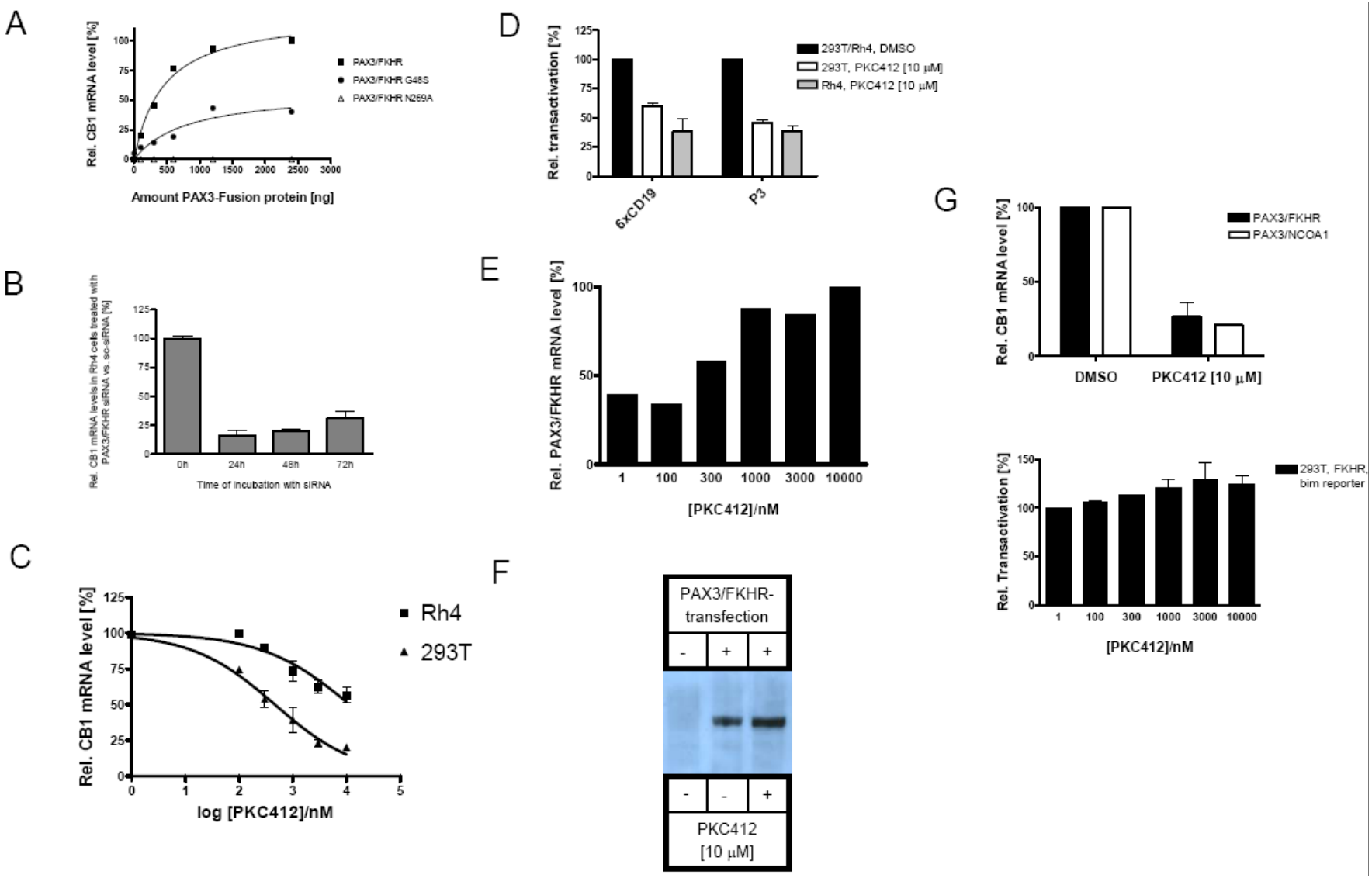
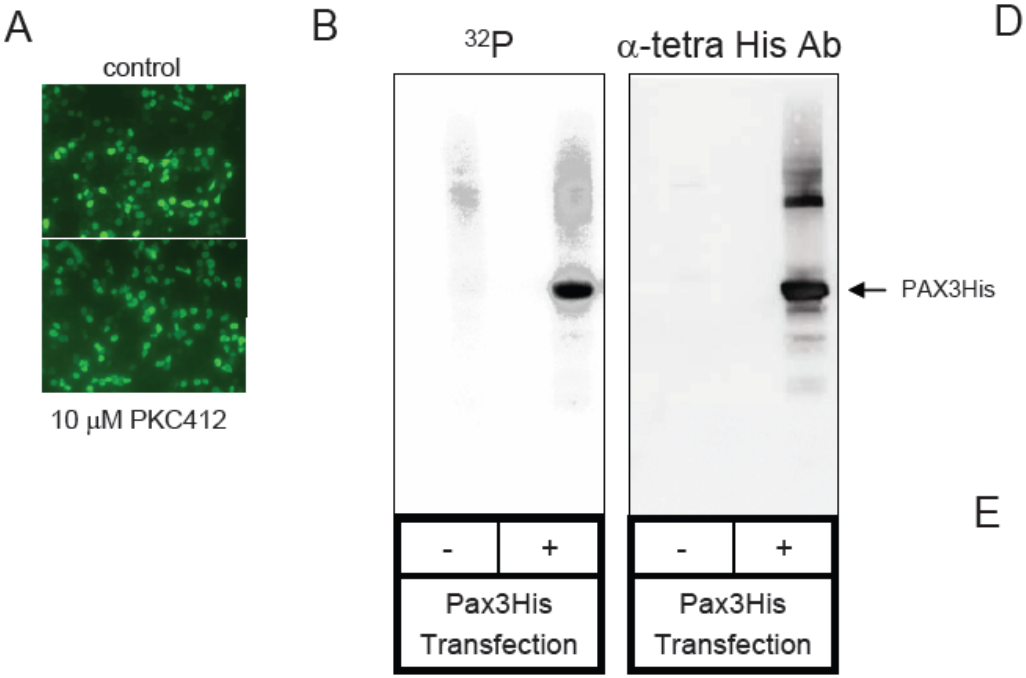
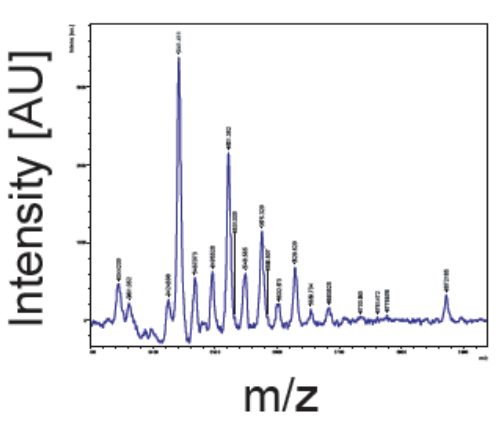
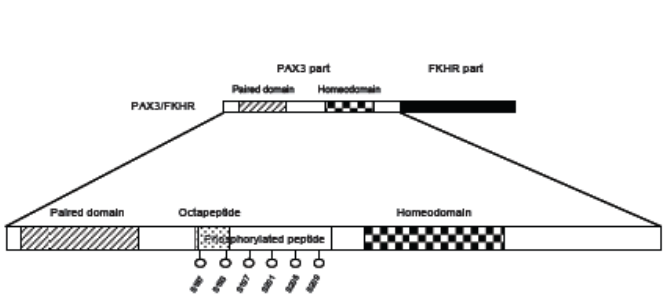
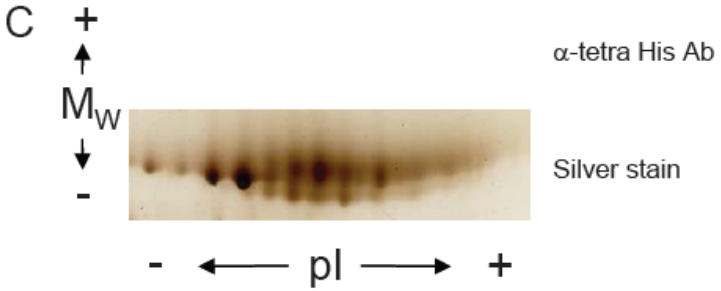

A
Rel. CB1 mRNA level [%]
PAX3/FKHR
PAX3/FKHR G48S
PAX3/FKHR N269A
Amount PAX3-Fusion protein [ng]
B
Rel. CB1 mRNA levels in Rh4 cells treated with PAX3/FKHR siRNA vs. sc-siRNA [%]
0h
24h
48h
72h
Time of incubation with siRNA
C
Rel. CB1 mRNA level [%]
Rh4
293T
log [PKC412]/nM
D
Rel. transactivation [%]
293T/Rh4, DMSO
293T, PKC412 [10 µM]
Rh4, PKC412 [10 µM]
6xCD19
P3
E
Rel. PAX3/FKHR mRNA level [%]
[PKC412]/nM
F
PAX3/FKHR-transfection
PKC412 [10 µM]
G
Rel. CB1 mRNA level [%]
PAX3/FKHR
PAX3/NCOA1
DMSO
PKC412 [10 µM]
Rel. Transactivation [%]
293T, FKHR, bim reporter
[PKC412]/nM

Fig.4


A
control
10 μM PKC412
B
32P
α-tetra His Ab
PAX3His
-
+
Pax3His
Transfection
-
+
Pax3His
Transfection


D
Intensity [AU]
m/z

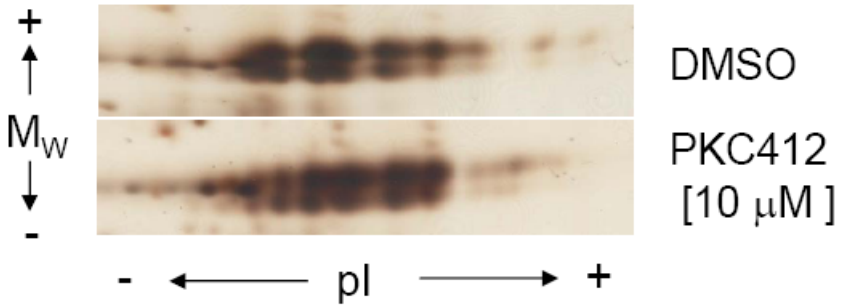

E
PAX3 part
FKHR part
Paired domain
Homeodomain
PAX3/FKHR
Paired domain
Octapeptide
Homeodomain
Phosphorylated peptide


C
+
Mw
-
α-tetra His Ab
Silver stain
-
pI
+

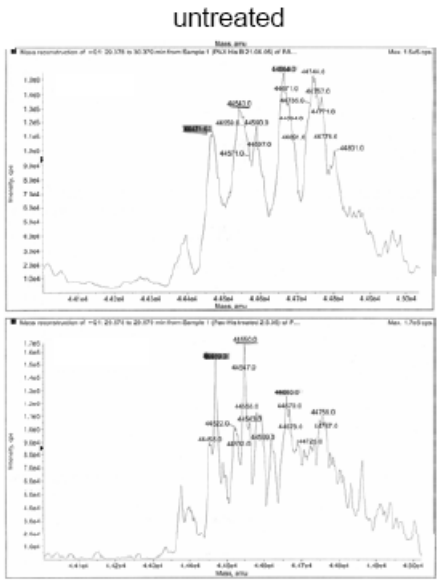

F
187
201
205
209
Human adlerkeaeesekkakhsidgilseras-apgsdegsdidsepdlplkrkq
Mouse adlerkeaeesekkakhsidgilseras-apgsdegsdidsepdlplkrkq
Zebrafish devekreieenerrakhsidgilgdrss---hsdegsdvdsepglplkrkq
Xenopus meldrkeqeesekrakhsidgilrerapvspeseegsdidsepdlplkrkq

Fig.5

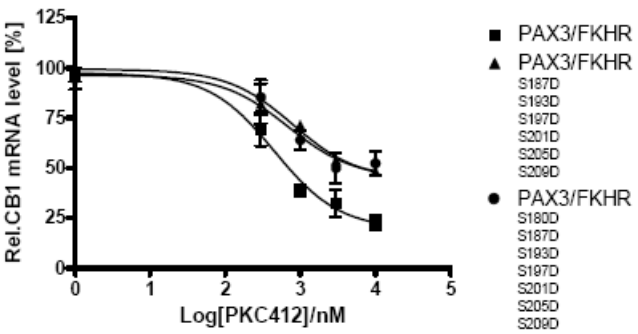
A


+
M_W
-
- pI +
DMSO
PKC412
[10 μM]

B


untreated
10 μM PKC412

C


Rel.CB1 mRNA level [%]
125
100
75
50
25
0
0 1 2 3 4 5
Log[PKC412]/nM
PAX3/FKHR
PAX3/FKHR
S187D
S193D
S197D
S201D
S205D
S209D
PAX3/FKHR
S180D
S187D
S193D
S197D
S201D
S205D
S209D

D

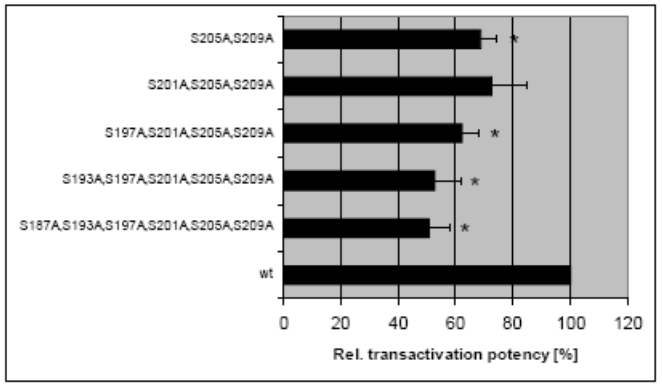

S205A,S209A
S201A,S205A,S209A
S197A,S201A,S205A,S209A
S193A,S197A,S201A,S205A,S209A
S187A,S193A,S197A,S201A,S205A,S209A
wt
0 20 40 60 80 100 120
Rel. transactivation potency [%]

Fig. 6 A:

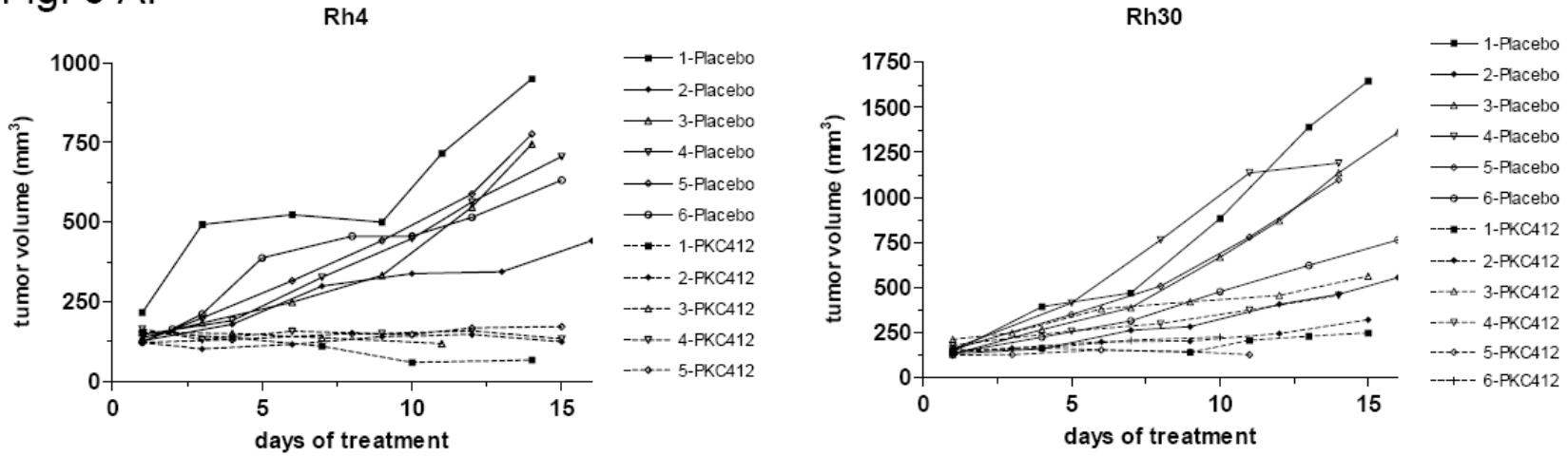

Rh4
tumor volume (mm3)
1000
750
500
250
0
0
5
10
15
days of treatment
1-Placebo
2-Placebo
3-Placebo
4-Placebo
5-Placebo
6-Placebo
1-PKC412
2-PKC412
3-PKC412
4-PKC412
5-PKC412
Rh30
tumor volume (mm3)
1750
1500
1250
1000
750
500
250
0
0
5
10
15
days of treatment
1-Placebo
2-Placebo
3-Placebo
4-Placebo
5-Placebo
6-Placebo
1-PKC412
2-PKC412
3-PKC412
4-PKC412
5-PKC412
6-PKC412

Fig. 6 B:

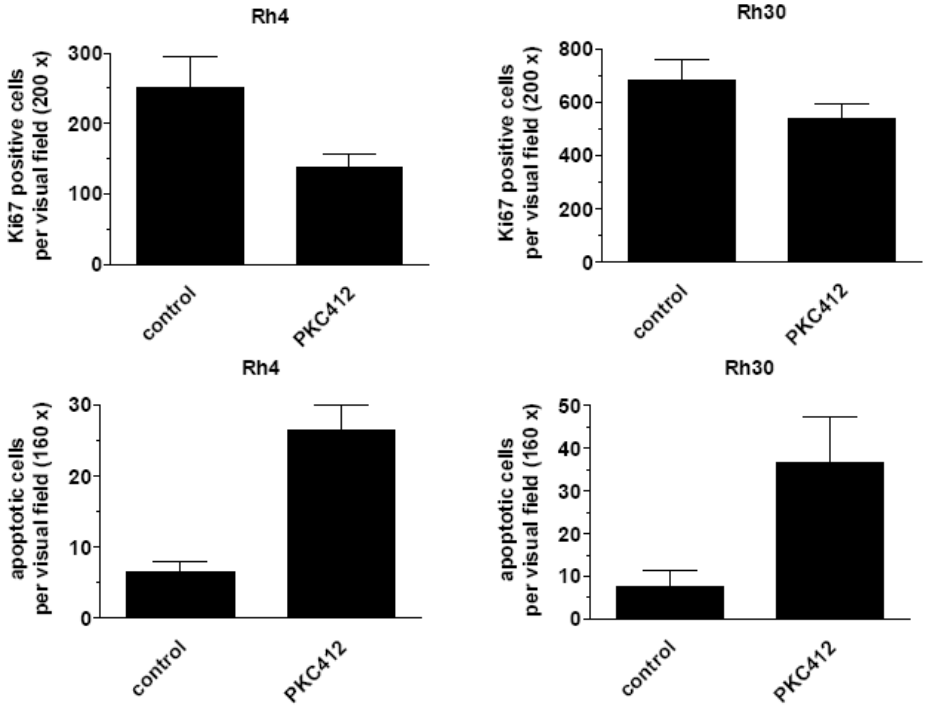
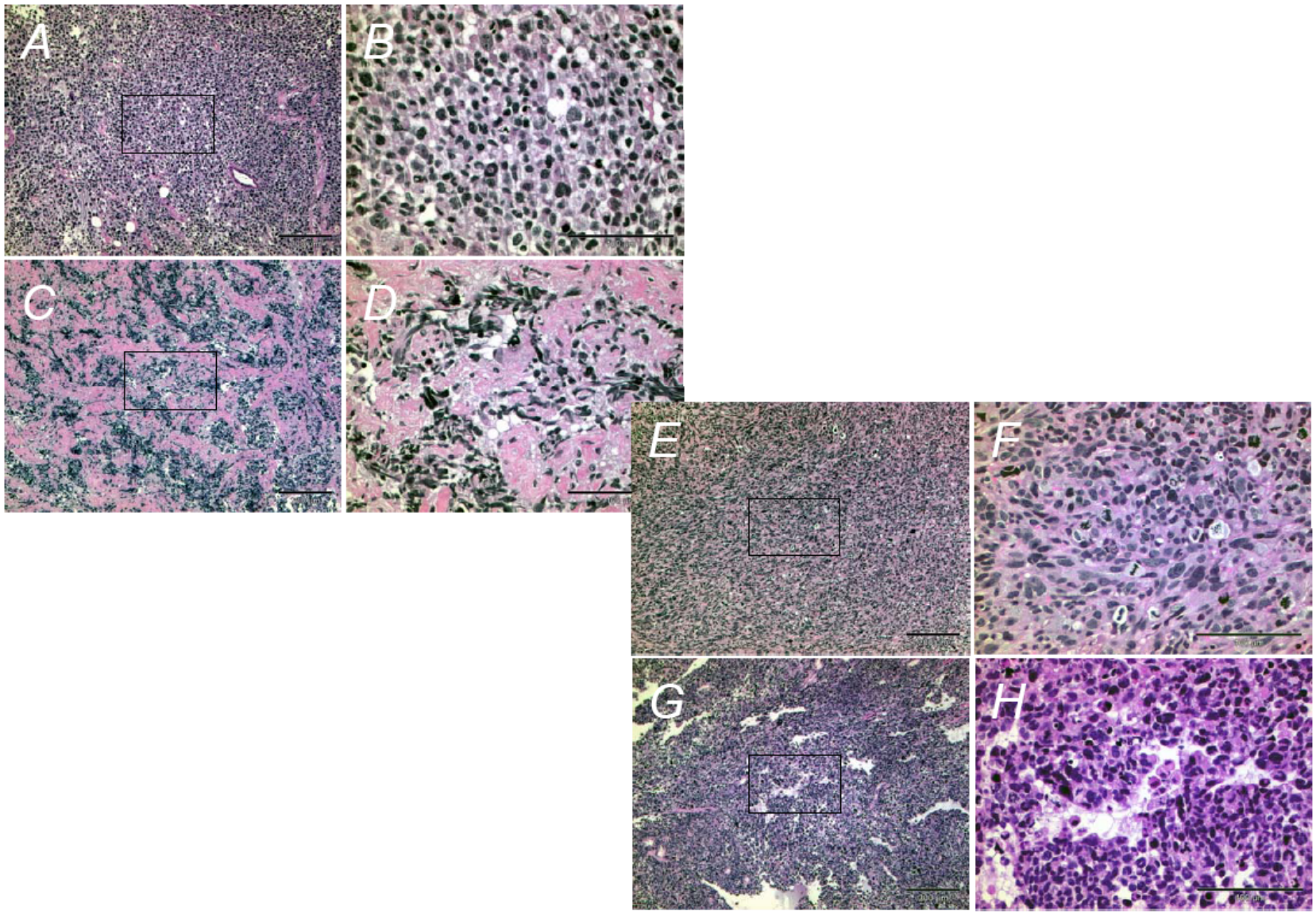

Rh4
Ki67 positive cells per visual field (200 x)
300
200
100
0
control
PKC412
Rh30
Ki67 positive cells per visual field (200 x)
800
600
400
200
0
control
PKC412
Rh4
apoptotic cells per visual field (160 x)
30
20
10
0
control
PKC412
Rh30
apoptotic cells per visual field (160 x)
50
40
30
20
10
0
control
PKC412

Fig. 6 C:



7 Additonal results

PAX3 role in melanoma cell survival

Margret Ebauer, Beat W. Schäfer

Introduction

PAX3 belongs to the group of (paired box) genes that encode a set of transcription factors. To date, nine members (PAX1-PAX9) have been described (Dahl et al., 1997; Stuart and Gruss, 1996) involved in regulation of tissue development and cellular differentiation in embryos. Apart from its role in embryonal development, different PAX family members have been assigned with a wide range of cancers (Muratovska et al., 2003). The precise role of PAX transcription factors in cancer is not known, but it has been shown that PAX genes are preventing terminal differentiation and maintaining progenitor cell state (Tremblay et al., 1996). Two members of PAX gene family, PAX3, and PAX7, show tumor-associated expression in different cancer types, among them neuroblastomas (Harris et al., 2002), squamos cell lung carcinomas (Racz et al., 2000) and rhabdomyosarcoma (Frascella et al., 1998). In rhabdomyosarcoma, PAX3 is implicated in the invasive and metastatic potential of cancer cells, as it plays a role in maintenance of a progenitor cell state and promotes cell survival. Similarly, the function of PAX3 at a nodal point in adult melanocyte stem cell differentiation has been described, where PAX3 simultaneously initiates melanogenic cascade while preventing terminal differentiation (Lang et al., 2005). This finding was supported by several studies demonstrating the “oncogene –addiction” of eRMS and melanoma tumor cells, as inhibition of PAX3 expression in either ERMS or melanoma cell lines using siRNA or antisense PAX3 oligonucleotides results in induction of apoptosis (submitted manuscript)(Bernasconi et al., 1996; Elbashir et al., 2001; Frascella et al., 1998; Scholl et al., 2001).

In our work, we aim to establish an RNAi based approach to reveal the role of PAX3 in the melanoma cell viability. Moreover, we examined the effect of PKC412, a kinase inhibitor, on melanoma cell survival to get insights into regulation of PAX3 activity.

Material and Methods

Cell lines

Mel15 and A365 melanoma cell lines were provided by Dr. R. Drummer (University Hospital Zürich, Switzerland). Cells were maintained under proliferating conditions in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and grown in 95% air, 5% CO_2 at 37^{o}C.

siRNA-mediated silencing

PAX3 knockdown was performed by use of the RNA interference (RNAi) technique (Elbashir *et al*., 2001). A total of 2x10^5 melanoma cells was plated and 24h later transfected with a combination of two chemically synthesized siRNAs (5'AAGAGAGAACCCGGGCAUG-dTdT and 5'CAUGGAUUUUCCAGCUAUA-dTdT) both targeting the PAX3 gene (Qiagen, Hombrechtikon, Switzerland). Transfection was performed according to manufacture's instructions using 3μl of Lipofectamine (Invitrogen, Basel, Switzerland) and 20nM siRNA (final concentration).

Quantitative RT-PCR

Total RNA samples (1μg) were reverse-transcribed with Oligo(dT)$_{15}$ Primer using the Omniscript Reverse Transcription Kit (Qiagen). Quatitative RT-PCR detection of PAX3 and GAPDH was performed with commercially avaible assays-on-demand Hs00240950_m1, and Hs99999905_m1 (Applied Biosystems, Rotkreuz, Switzerland), respectively. TaqMan analysis was carried out according to the manufacturer's instructions on an Applied Biosystems 7900HT Sequence Detector. Expression levels of the gene of interest were normalized with GAPDH expression levels. Experiments were performed in triplicate and standard deviations were calculated based on the results of three biological replicates.

Cell Proliferation assays

Cell proliferation was measured using the MTT assay system (Roche, Rotkreuz, Switzerland). A total of 1x10^4 Mel15 or A365 cells were plated per 96-well and transfected 24h later. The amount of converted MTT reagent was measured at different time points up to 72h later by a multi-detection microplate reader (Bio-Tek Instruments, Inc., Littau, Switzerland) at wavelength 595 nm.

For the measurement of cell proliferation upon treatment with kinases inhibitor, the compound PKC412 was used (kindly provided by Novartis, Basel, Switzerland). A total of 1x10^4 Mel15 or A365 cells were plated per 96-well and treated 24h later with PKC412 in a final volume of 100 μl medium including 10% serum for 72 hrs.

Results

1. PAX3 promotes cell survival of melanoma cells

The cell survival promoting role of PAX3 in eRMS and PAX3/FKHR in aRMS is well characterized. However, wide range of different cancer cell types also show high expression of PAX3, among them cells from brain, melanoma, breast and colon cancer (Muratovska et al., 2003). Previously, the role of PAX3/FKHR and PAX3 in aRMS and eRMS development and maintenance was shown using a siRNA-mediated down-regulation strategy (manuscript submitted). As this approach has yet not been used in melanoma cells, we sought to characterize the role of PAX3 in melanoma cell lines by siRNA-mediated down-regulation. We used the melanoma cell line Mel15 which expresses PAX3 at high levels and A365 cells, which do not express PAX3.

The combination of two published siRNAs used for PAX3 and PAX3/FKHR silencing was also applied in this approach. The treatment with specific siRNA suppressed the PAX3 mRNA level in Mel15 melanoma cells by nearly 70% (Figure 1A). In the control cell line, GAPDH mRNA level was suppressed by treatment with siRNA specific for GAPDH by approximately 50% (Figure 1B).

To characterize the physiological effects occurring upon siRNA treatments, we next measured proliferation of Mel15 and A365 cells after treatment with PAX3 specific and control siRNAs for 24hrs, 48hrs and 72hrs. Cell growth was found to be inhibited in Mel15 cells treated with siRNA targeting PAX3, whereas only unspecific inhibition of proliferation rates was observed in A365 cells (Figure 1 C, D). Thus, an anti-apoptotic function of PAX3 in Mel15 could be demonstrated. These results are in accordance with earlier findings, demonstrating an anti-apoptotic function of PAX3 melanoma cells treated with specific antisense oligonucleotides (Scholl et al., 2001) and therefore validate our siRNA approach also on the physiological level.

2. PKC412 treatment leads to proliferation stop of melanoma cells

It has been shown in different tumors types that a constitutive active signaling cascade is contributing to tumorigenesis. For example, in alveolar rhabdomyosarcoma, PKC412 has been shown to regulate PAX3/FKHR activity by altering its phosphorylation status. Moreover, an antitumorigenic potential of PKC412 for aRMS has been shown *in vitro* and *in vivo* (Wachtel, Amstutz et al., manuscript in preparation). We assumed that similar regulation of PAX3 transcription factor by kinases could occur in melanoma cells. To investigate that, we measured the effect of PKC412 treatment of melanoma cells Mel15 and A365. Indeed, PKC412 treatment of Mel15 inhibited the proliferation rate in dose-dependent manner (Figure 2A), whereas A365 cells did not show any PKC412 specific effect (Figure 2B). Thus, the regulation of PAX3 activity by protein kinases seems to play a role in the survival of the two examined melanoma cells.

Discussion

PAX3 is a transcription factor which activates different tissue-specific targets and appears to play an essential role in the development of both, melanogenic and myogenic cell lineages. The oncogenic properties of PAX3 include the capability of promoting cell viability, migration and self-sufficiency in growth signals. For the understanding how these oncogenic characteristics are mediates, identification of the relevant PAX3 downstream targets and signalling pathways involved in tumor development are required. However, the set of PAX3 target genes, which are involved in these properties remains, with the exception of some single gene as c-met (Relaix et al., 2003) and some signalling pathways involving HGF/SF (Saitoh et al., 1994) and IGF-1R receptors (Furlanetto et al., 1993) are largely unknown.

The RNAi strategy revealed the promoting role of PAX3 in cell survival of melanoma cells. PAX3-expressing melanoma cell line was susceptible to siRNA treatment and showed reduced cell proliferation compared with control PAX3-negative melanoma cells. Similar results were achieved in the previous work performed with RMS cell lines (submitted manuscript), suggesting an antiapoptotic function of PAX3 in both, melanoma and rhabdomyosarcoma. The antiproliferative effect of PKC412 in melanoma cells suggests that the activity of PAX3 might be regulated via pathways involving protein kinases. However, these preliminary results need to be verified by further studies.

Taken together, the silencing of PAX3 by siRNA or inhibition of PAX3 transcription factor activity by broad spectrum kinase inhibitors such as PKC412 might represent a possible novel therapeutic strategy for treatment of melanoma.

Figure 1

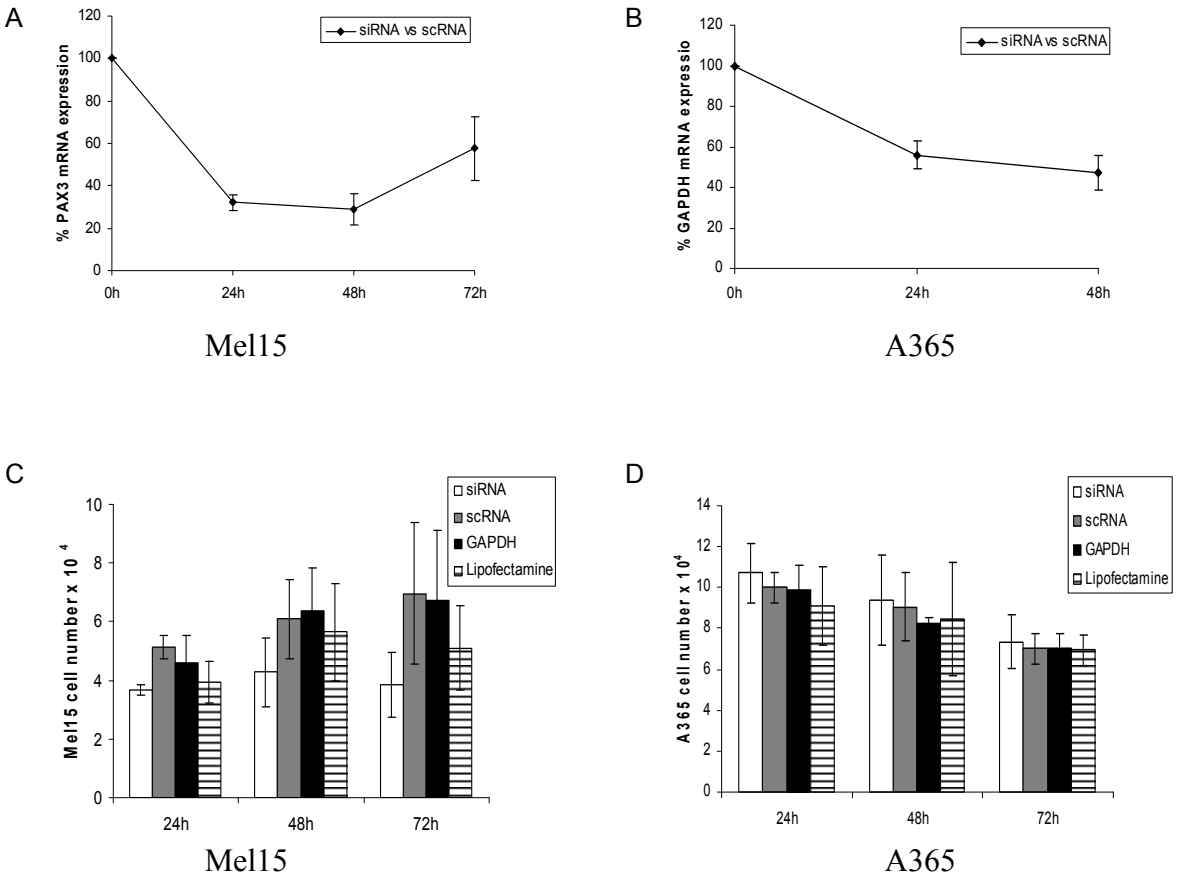

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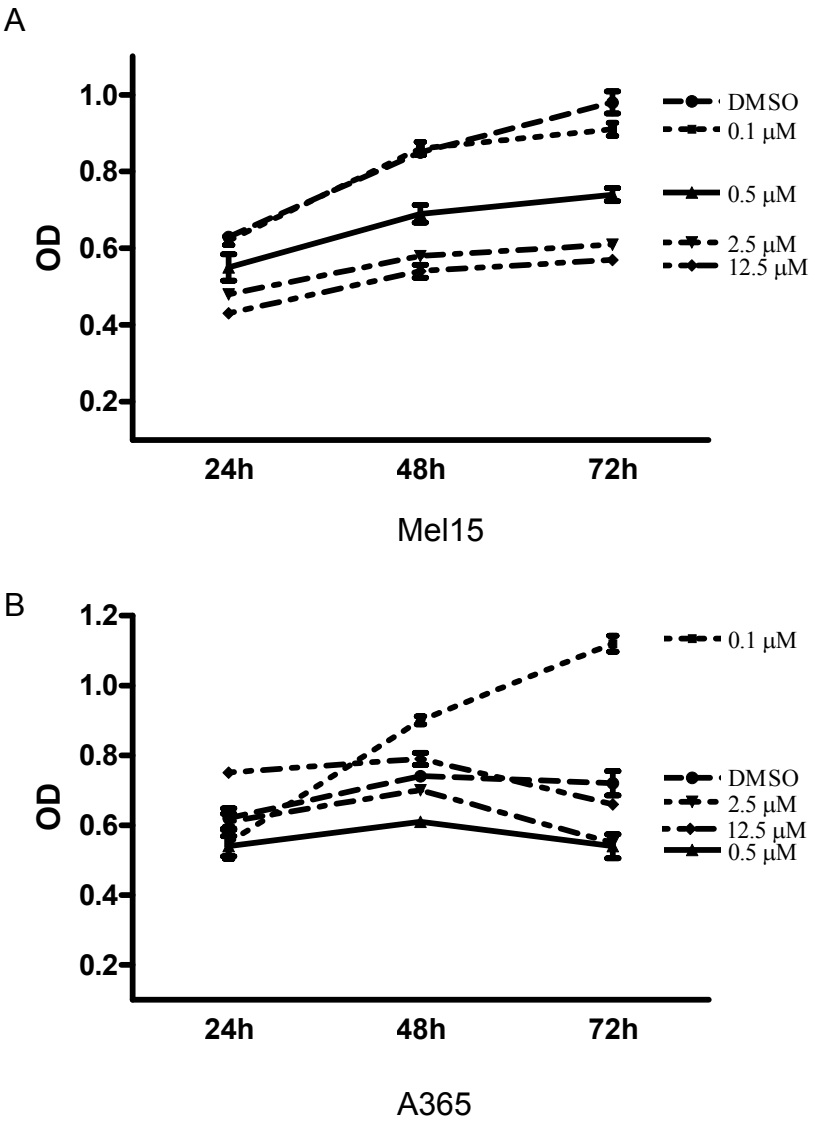
Figure 2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DMSO
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A365

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8 Discussion

8.1 RNAi technique

RNA interference (RNAi) is a process of sequence specific post-transcriptional gene silencing. The mechanism of RNAi has been first recognized by Andrew Fire et al. in 1998 and already in 2002 the phenomenon or RNAi was termed as the “Breakthrough of the Year” by the journal *Science*. RNAi is initiated by the enzyme called DICER, which processes double-stranded RNA (dsRNA) into small pieces of 21-25 nucleotide small interfering RNA (siRNA) (Bernstein et al., 2001). The siRNAs are incorporated into the RNA-induced silencing protein complex (RISC), a multicomponent nuclease, and guide this complex to the target mRNA which is then recognized and cleaved. This guidance of RISC to target mRNA is highly sequence specific and, thus, target specific. RNAi regulation of endogenous genes in mammalian cells occurs via short dsRNA molecules termed as microRNA (miRNA). MiRNAs are a class of non-coding RNAs of approximately 22 nucleotides. MiRNA can not only inhibit gene expression by mRNA degradation, but also inhibit the translation of target genes.

Although RNAi is a naturally occurring process, it is nowadays considered as one of the most powerful and indispensable tools for gene silencing in molecular biology. Using short double-stranded RNA (dsRNA) molecules, essentially every gene in the genome can be selectively silenced, thus enabling the study of gene function in development, intracellular signaling, cancer, infection and more in endogenous context. By “knocking down” a gene with RNAi and determining the cellular responds, significant insight into the function of the gene can be gained.

8.2 RNAi in cancer

In different tumor cell models, various individual genes have been silenced with the use or RNAi technique and their knockdown led to significant physiological changes. Among these genes were genes involved in tumor development like oncogenes, growth factor receptor genes, genes encoding for anti-apoptotic molecules, signaling molecules, telomerase and others. The physiological effect of such genes for tumor survival can be then clearly determined in endogenous context. Some examples of this approach is the RNAi-mediated silencing of several members of Ets transcription factor family, BCR-ABL in CML or EWS-FLI1 in Ewing’s sarcoma. The downregulation of BCR-ABL and EWS-FLI1 resulted in apoptosis and inhibition of tumor cell growth (Wohlbold et al., 2003; Scherr et al., 2005, Hu-Lieskovan et al., 2005). Another important transcription factor family involved in cancer development is myc transcription factor family. A member of this family, c-myc was downregulated in breast cancer cells, which also lead to apoptosis and inhibition of cell growth (Wang et al., 2005). Similarly, in our work, the antiapoptotic effect of PAX3/FKHR in aRMS and PAX3 in eRMS cells could be demonstrated using siRNA (manuscript under revision).

Moreover, endogenously expressed classes of siRNAs, miRNAs, have global effect on gene expression and may inhibit oncogenes or tumor-suppressor genes, thus itself acting as tumor suppressors or oncogenes. This hypothesis was supported by the finding, that more than 50% of genes encoding for miRNAs were localized in cancer-associated genomic regions or in fragile sites (Calin et al., 2004). Indeed, tumor suppression function of miRNA could be determined in CLL (Calin et al., 2002) and the oncogene function of miRNA was reported for B-cell lymphomas (Eis et al., 2005), Hodgkin's lymphomas (Kluvier et al., 2005) and human breast cancer cells (Iorio et al., 2005).

8.3 RNAi as tool for functional genomic

The extraordinary selectivity of RNAi quickly made it the tool of choice for functional genomics. In this approach, siRNA-mediated gene silencing can be coupled with microarray screening and putative targets of the silenced genes can be identified. Furthermore, systematic pathway analysis can be performed with microarray data to determine of the role of the silenced gene in intracellular pathways. Thus, determination of what a gene product does and with what other products it interacts is faster and easier combining these techniques.

Moreover, identification of downstream target genes of distinct cancer-associated transcription factors may help us to understand the role of the fusion products in tumor development. Previously, downstream targets were mainly identified by transfection of fusion genes into different cell lines followed by microarray analysis to identify differentially expressed genes. The opposite approach, previously achieved by generation of loss-of-function mutants through different methods like chemical mutagenesis or expression of anti-sense library (Deiss and Kimchi, 1991), became a faster and more specific with the development of RNAi techniques. There are numerous studies determining various downstream targets of transcription factors associated with cancer, as for example EWS-FLI1 in Ewing's sarcoma (Prieur et al., 2004) or c-myc in breast cancer cells (Cappellen et al., 2007). However, not all of the putative targets identified by this approach turned out to be true when analysed in vivo.

Similarly, we sought to identify target genes of the chimaeric transcription factor PAX3 and PAX3/FKHR combining RNAi technique with microarray analysis. In order to identify target genes relevant in vivo, we compared the microarray data obtained in cell culture to data acquired from tumor biopsies and to published microarray data derived from experiments performed with different cell lines. Moreover, direct targets had to be distinguished from unspecific effects caused by RNAi or secondary effects based on the gene expression changes in response to the knockdown of the target.

In our work, we performed a time course assuming that direct target genes are the first genes showing different expression. Indeed, we could discriminate between direct and indirect effects and thus conclude whether the identified gene is a direct or indirect target. In our case, we could identify 51 putative target genes of PAX3/FKHR which are relevant in vivo and one of them, TFAP2beta, was characterized as a direct target of PAX3/FKHR. We validated the microarray data by additional methods, as the direct binding of PAX3/FKHR to the promoter region of TFAP2beta was shown by promoter studies and the physiological link between the two genes was confirmed by rescue approach. Moreover, PAX3 and

PAX3/FKHR targets that play a role in other oncogenic pathways, in our case the repression of myogenic differentiation or enhanced tissue invation and metastasis could be detected by microarray analysis. However, the challenging issue in this field is the correct analysis, interpretation and validation of microarray data and finally, a functional link between discovered genes and the observed phenotype should be provided.

8.4 RNAi therapy

The use of RNAi technique as novel therapy for clinical applications is a promising approach. The great advantage of treatment with small interference RNA (siRNA) is the selectivity of which siRNA destroys its target without affecting other genes. Wide range of clinical applications are imaginable as any gene whose expression contributes to disease is a potential target for therapy, from oncogenes in cancer therapy to genes responsible for heart disease, Alzheimer's disease, diabetes, and more.

Several biotech companies are already developing RNAi-based therapy, one of them, called Sirna Therapeutics aim to deliver siRNAs to key cellular targets through systemic, local and topical applications. Sirna's most advanced programs are in age-related macular degeneration AMD, viral hepatitis, respiratory disease and dermatology. Sirna is also developing programs in diabetes, Huntington's Disease and oncology. Currently, different siRNA molecules are in preclinical phase and some of them are even tested in phase I and phase II clinical studies. For example, siRNA for Hepatitis C is in phase I clinical study and ongoing phase II clinical study with siRNA for AMD has been published.

However, there are technological hurdles need to overcome and the biological limitations need to consider for achieving effective therapeutics. The main prerequisite for successful siRNA therapy is generation of a stable molecule which can be efficiently delivered to its target cells. Furthermore, unspecific significant toxic effects or off-target effects neither in target nor in non-target tissue should be caused by the therapy. Therefore, to use siRNA as a compound, chemically optimized siRNA's are needed because charged oligonucleotides would not pass through a lipid layer and unmodified RNA would be rapidly degraded in the bloodstream or excreted by the kidneys. Complexes of RNA with a lipid of backbone modifications have been developed in order to increase cell uptake and to improve stability in the blood stream.

The delivery of siRNA is another major problem of RNAi therapy approach. To overcome this problem, nanoparticle delivery was developed by biotech companies. Therefore, novel nanoparticles were used that can deliver siRNAs at low doses to the cells of choice by local, systemic or topical delivery. However, in the phase II clinical study presented by the Sirna Therapeutics company, antisense has been applied in the eye. In other situations, like in cancer, delivery is more of an issue. Moreover, most effective antitumor drugs have multiple mechanisms of action. This has been shown with two other RNA-based therapies, antisense and ribozymes, both of which were promising tools for therapy but did not achieve this success. Therefore, it is possible that in some cases, the overspecificity of the RNAi could be even disadvantageous for the therapeutical outcome. Other obstacles for the RNAi therapy might be the toxicity level and the therapeutic effect of treatment. The target of interest may be in normal cells as well as

cancer cells. And as the number of RISC complexes in the cell is unknown, if the amount of RNA needed to have a therapeutic effect may occupy all the available complexes. RNAi is a natural cell system that is there for knocking out endogenous gene function. With the introduction of foreign RNA, will the system continue to perform its normal function as well, or will it become saturated.

However, if these hurdles can be resolved, RNAi could revolutionize the identification and validation of new drug targets and with the development of RNAi as therapeutic agents personalized disease treatment could be achieved.

9 Conclusions and Outlook

There are two main areas of RNAi applications: drug discovery and research, and potential therapeutic applications. In our work, we could prove that RNAi can be used for characterization of gene functions and as functional genomics tool for identification of new target genes. However, the entity of oncogenic pathways involved in RMS formation and progression still remains to be clarified.

In our work, a “gene by gene” method was chosen, in which individual transfection for silencing of a distinct gene has been performed. Recently established novel high-throughput systems however allow large-scale analyses of gene function. Large collections of synthetic siRNAs have been developed, some of them targeting smaller set of genes of interest, like various kinases and phosphatases. But also whole-genome libraries have been developed which consist of at least 3 distinct siRNA molecules specifically targeting virtually all annotated genes in the human, mouse and rat genomes.

In order to study multiple cellular processes, protein detection technologies have been adopted by large-scale screening platforms, with analogy to the analysis of gene expression using DNA microarrays. Thus, novel cell microarrays have been developed enabling robust and automated transfection in order to be able to introduce the siRNA molecules under constant conditions in mammalian cells. In this RNAi microarray platform, a transfection matrix is first arrayed on glass slides overlaid with a monolayer of adherent cells which take up siRNA molecules that had been deposited on the slide before cells were plated. This technique of reverse transfection combined with large-scale RNAi libraries allow the measurement of effects of gene silencing by digital image analysis by specific fluorescent monoclonal antibodies at a single cell level. Selective silencing of any gene in the genome combined with high-throughput functional genomics studies enables identification of gene functions on a whole genome scale which could be a future method to screen for potential therapeutic targets associated with diseases. Moreover, a database of gene expression signatures for various perturbations such as siRNA-mediated gene knockdowns could be created in future. This database would then be used as a reference to analyze new profiles obtained for novel inhibitors, thus providing value in drug target identification and candidate compound selection.

Despite the fact that the use of genome-wide RNAi libraries is a new technology, this approach holds great promise for better understanding of cancer-related processes and the consequences of alterations in these processes in different cancer types, among them RMS. In future, using the RNAi libraries and large-scale platforms, we could use RNAi cell arrays for identification of further target genes of transcription factors. Predicted promoter regions can be cloned in vectors upstream of reporter genes, and cell lines stably expressing the reporter construct can be subjected to genome-wide siRNA arrays.

Because of its exquisite specificity and efficiency, RNAi is an important tool not only for functional genomics, but also for gene-specific therapy. Genome-wide screens using RNAi can help to identify components of functionally related pathways and therefore be used for the development of new and improved therapeutic targets. Thus, combination of microarray technology and cellular biology methods should in the future provide additional tool for development of robust RNAi-based screening methods and

consequently toward efficient drug target selection and evaluation. Discovery of new drug targets by microarray and determination whether reducing its gene expression is likely to be therapeutically useful makes then the validation of potential drug targets faster more specific and thus, more effective. This is especially important for aggressive tumour types like aRMS. Although the conventional treatment of RMS with chemo- and radiotherapy has been greatly improved, the 5-year survival rate is still quite low (30% for aRMS, 60% for eRMS). Especially for the more aggressive aRMS subtype, new therapy forms are required, as aRMS cells become resistant to conventional chemo- and radiotherapy. Targeting of the mRNAs of cancer-related genes would allow a specific and efficient treatment of RMS, therefore more detailed investigation of RNAi against clinically relevant gene targets in appropriate model systems should be performed.

10 Literature

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11 Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myelogenous leukaemia
ARMS	alveolar rhabdomyosarcoma
cDNA	complementary DNA
CDK	cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CML	chromic myelogenous leukaemia
EMSA	Electrophoretic Mobility Schift Assay
ERMS	embryonal Rhabdomyosarcoma
EWS	Ewing's sarcoma
FKHR	forkhead related gene
HLH	helix-loop-helix
HM	Homeodomain
LZ	Leucine zipper
MDS	myelodysplastic syndrome
MTTassay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
PAX3	Paired box 3
PD	Paired domain
Rb	Retinoblastoma
RMS	Rhabdomyosarcoma
RT-PCR	reverse transcriptase polymerase chain reaction
scRNA	scrambled RNA
siRNA	small inhibitory RNA
T-ALL	T-cell acute leukaemia
TFAP2b	Transcription factor activating enhancer binding protein 2 beta

12 Acknowledgements

I would like to express sincere thanks to Beat Schäfer for giving me the opportunity to work on this project. I would also like to thank you for the supervision and for proofreading and refereeing my thesis.

I also want to thank Felix Niggli for co-refereeing my thesis and I'm greatful to Joe Jiricny for supervising my thesis.

Marco Wachtel, thanks a lot for showing me almost everything I know about working in the lab and for the challenging discussions we often had. I also want to thank you for reading and correcting this thesis.

Further, I thank everybody in the lab for the great atmosphere we always had!
Katarina Hajdin ("Kat") and Alexa Burger (also known as the "hunnybunies"), Kathya Pretre ("Kleines"), Ludwig Zaunder ("Ludi"), Susanne Oesch ("Sue"), Marcus Dorner ("Dorni"), Rahel Schaub ("Rajelita"), Laura Bonapace, Markus Rechsteiner ("PN") - thank you all for the great friendship and the big fun we always had during these years, also outside of the lab.

Beat Bornhauser (BB), Torsten Heineke (for once, just Torsten), Michele Bernasconi, Jürg Sigrist, thank you for the patience and assistance.

13 Curriculum vitae

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